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THE ROLE OF THE SPLEEN AND THE DYNAMICS OF HEMOLYSIN PRODUCTION IN HOMOLOGOUS ANAMNESIS

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Our previous studies on the dynamics of hemolysin production in rabbits after single (1950) and multiple (1951) intravenous injections of sheep red cells have been extended to include a study of the homologous anamnestic response following single and multiple reinjections with the same antigen into intact rabbits and into rabbits splenectomized either at the time of the initial injection or of the reinjection. After an induction period, hemolysin begins to be produced rapidly at one continuous rate of accumulation (cf. fig. 8B) or at two (cf. fig. 12) or more rates to peak titer and thereafter generally declines at several varying rates. Our results indicate that peak antibody titer after both the initial rise and anamnestic rise is due chiefly to the activity of the spleen after a single injection of antigen and is due, relatively, more to the activity of non-splenic sources after repeated injections of antigen.

A review of the literature and a discussion of the differences between primary and secondary antibody responses are given by Burnet and Fenner (1949). Pertinent work will be reviewed in the discussion.

MATERIALS AND METHODS

Materials and methods have been previously described (1951 and especially 1950). The following brief résumé will give only necessary orienting facts.

Of the ten series, the following were performed simultaneously: 1 with 2, 5 with 9, and 6 with 7 and 10. Series 3, 4 and 8 were tested in groups of 6 to 12 rabbits. Splenectomy was usually performed on the day of the initial injection or reinjection and on the day of the first of a series of initial injections or reinjections. In series 8, the rabbits were splenectomized before or after the initial injection as shown in figure 14. In table 1B, Student's method of paired comparisons was used to obtain the statistical significance of the differences for all series except series 8.

Three standardized amounts of sheep red cells were used exclusively for all injections into rabbits. All were given intravenously per kg body weight. Individual injections for a given rabbit of the first and second immunization, therefore, whether of a single or a multiple series of injections, were always relatively but not absolutely the same. Suspensions of red cells were standardized with a Klett-Summerson photoelectric colorimeter by determining the hemoglobin released by distilled water hemolysis. These standardized amounts may be approximately designated for convenience as small (equivalent to 1.25 ml 0.1% or 2×10^7 cells), intermediate (equivalent to 1.25 ml 1% or 2×10^8 cells) and large (equivalent to 1 ml 10% or 1.6×10^9 cells), respectively. In experiments 1, 3, 4 and series 9 and 10, the red cells for injecting and testing were from the same sheep.

Hemolysin titers in 50% hemolytic units were determined by plotting the amount of serum against the degree of hemolysis (ascertained photometrically) according to von Krogh's equation. They are expressed interchangeably in four ways: In ml of serum, as the reciprocal of titer, as the dilution of serum contained in 1 ml, or as the number of 50% hemolytic units per ml.

We have previously given detailed evidence (1950 and 1951) that the rise and fall of antibody titers after single or multiple antigen injections fit a series of discontinuous curves described by the first order equation:

$$A_t = A_0 e^{kt} \quad (1)$$

in which A_0 and A_t are antibody titers at times

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zero and t, respectively, and k is the constant of increment. Fitted titers and fitted peak titers were obtained from these lines. Declines in hemolysin titer are indicated both as $-k$ and as the apparent half life in days ($t_{1/2}=0.693/k$) to facilitate comparisons. The true half life of antibody probably approximates 5.5 days with $k=-0.126$ (cf. our earlier work, 1950).

To compare the first and second antibody responses, we have used (1) antibody accumulating in the serum at peak titer together with the time and rate of rise (k) and (2) the total amount of antibody formed in 30 days taking into account the antibody undergoing metabolic decay. In characterizing the accumulation of antibody to peak titer, we have used (a) the induction period, i.e., the number of days after the first antigen injection during which the preimmunization titer is not exceeded, (b) the total number of days to peak titer after the first antigen injection, and (c) the amount of antibody in the serum. Three methods are used to indicate the amount of antibody and are described in the following paragraphs.

The amount of antibody in the serum at peak titer is represented graphically for all series in figures 3, 4, 6, 7, 9 through 11 and 14 through 16. In these figures, peak titers were plotted arithmetically after both injections with the base of the bar of the second response at the level of the serum titer at the time of the second antigen injection. This method is simple, gives a direct over-all basis of comparison and was used in our earlier work. It is not adequate, however, to compare antibody responses when the second response is superimposed on a changing amount of antibody from the first one (cf. the second method below) or to compare total amounts of antibody formed including that lost from the serum by metabolic decay (cf. third method below). In addition, it does not take into consideration rates of accumulation.

The second method involved a derived peak titer and a derived rate of accumulation for the anamnestic response. These may be briefly explained as follows: The slope of the antibody curve from the first antigen injection was assumed to continue after the second injection throughout the initial rise to peak titer. A derived peak titer of the second response was thus considered to be the actual peak titer minus the assumed titer of the primary response plus the "normal" titer, i.e., the titer before the initial injection. In a few cases when the amount of antibody remained constant, this method necessitated the assumption of 5.5 days as the half life of the disappearing antibody of the primary response and is in error to the extent that this figure may be wrong.

In comparing the speed of the initial antibody response to peak titer, a derived single value of k was obtained for all primary responses involving two or more rates of accumulation and for all secondary responses which were superimposed on appreciable amounts of antibody from the primary response. The derived value of k for all primary responses was obtained from a line connecting the "normal" titer at the end of the induction period to the peak titer. The derived value of k for all secondary responses was obtained from a line connecting the "normal" titer placed at the end of the second induction period, i.e., where the second initial rise started, to the derived peak titer (see above) which was placed at the time when the actual peak titer of the reinjection was reached. Mean values for derived peak titers and derived rates for all series obtained by this second method are tabulated in table 1.

The total amount of antibody by the third method was calculated for 30 days during both primary and secondary responses because this length of time included the rise and most rapid decline after a single antigen injection and the titer of the primary response at the end of this period was so low that it could be disregarded in calculating constants for the secondary response. Total antibody for 60 days was also calculated after repeated injections. For each change of rate in antibody accumulation the following equations were used:

For each rise,

$$S_t=A_0\frac{k_1+k_2}{k_1}(e^{k_1t}-1) \tag{2}$$

(equation 9 of our earlier paper, 1951) where S_t represents the total amount of antibody formed at time t (serum antibody plus the antibody lost by metabolic decay); A_0, the observed serum antibody at time zero; k_1, the rate of antibody rise; and k_2, the rate of metabolic decay which we assume to be -0.126 or $t_{1/2}=5.5$ days (see our earlier paper, 1950).

In a plateau, where $k_1=0$,

$$S_t=A_0k_2t \tag{3}$$

from

$$\lim_{k_1\to 0}\left[A_0\frac{k_1+k_2}{k_1}(e^{k_1t}-1)\right]=A_0k_2t. \tag{4}$$

For each decline, where $k_2<0$ and >-0.126,

$$s_t=a_0\frac{k_2-k_2}{k_2}(1-e^{-k_2t}) \tag{5}$$

(equation 19 of our earlier paper, 1951) where s_t represents the total antibody formed at time t; a_0, the serum antibody at time zero; k_2, the rate of metabolic decay, which we assumed to be -0.126 as above; and k_3, the rate of serum antibody decline. Whenever $k_2 \leq -0.126$ (which gives $t_{1/2} = 5.5$ days or less), it is assumed that no antibody is being formed. Data obtained by this third method are shown in figure 18.

EXPERIMENTAL RESULTS

Data on 191 rabbits were used in characterizing the initial and anamnestic responses. Of these, 110 rabbits involved single or multiple reinjections into (1) 48 nonsplenectomized rabbits, (2) 30 rabbits splenectomized when reinjected and (3) 32 rabbits splenectomized at or near the time of the initial injection. Nine and seven of the rabbits in the first two categories, respectively, had served as operative controls for the initial injection of red cells for previous papers (1950 and 1951) and had had a testis, a kidney or the appendix removed on the day they were first injected with red cells. The work was divided into ten series within five experiments. The first two experiments deal with one injection and one reinjection of antigen, the fourth experiment with a repeated series of injections both initially and secondarily, and the third experiment with a repeated series of injections initially and one reinjection. The compensatory function of nonsplenic sites is studied in the fifth experiment. The intermediate or large amounts of antigen were used except for three rabbits in series 1. Each rabbit was always reinjected with the same relative amount it had received initially. Some rabbits were given a second single test reinjection a month or more after the first reinjection and 1 rabbit was given a third test. The rabbits usually weighed from 2 to 2.5 kg when initially injected and from 2 to 5 kg when reinjected. Partial data and graphs of some of the initial antibody responses have already been reported under identical rabbit numbers (1950 and 1951). In experiments 1, 3 and 4 and in series 9 and 10, the even-numbered rabbits were splenectomized and the odd-numbered ones were not.

Experiment 1 (series 1 and 2). A single reinjection of 1.25 ml of 1% sheep red cells into pairs of intact and splenectomized rabbits

This experiment was planned (1) to study the anamnestic response in intact rabbits (series 1) during the decline in hemolysin from peak titer and (2) to ascertain the effect of splenectomy (series 2). Rabbits were reinjected once from seven days to 109 days after a single initial injection. The experiment is illustrated by figures 1 through 5 and mean values may be found in tables 1 and 2. As compared to the initial peak titer, the mean peak titer after reinjection was not significantly different from that in intact rabbits but was markedly less in splenectomized animals, whereas the mean induction period after reinjection was significantly shorter in intact rabbits but not in splenectomized ones. In both groups, peak titer was reached sooner and at a faster rate.

Series 1 (16 intact rabbits).—Thirteen rabbits (odd numbers between 125 to 147 and 91) were injected initially with 1.25 ml 1% sheep red cells/kg and were each reinjected once with the same amount. A few were reinjected again after a month or more. In addition, three rabbits (73 to 75), injected initially with 1.25 ml 0.1% sheep red cells/kg, were each reinjected twice with a similar amount 109 and 141 days after the initial injection. Titers at the time of reinjection in all rabbits varied from 1:110 (147) to 1:5600 (120). The range was wide because some rabbits were reinjected at or just after peak titer.

TABLE 1.—*Mean values† for the primary (I) and secondary (R) responses in the same rabbit in section A and differences‡ between these values in section B.*

Series		Number of rabbits in group	Length in days of: Induction period	Length in days of: Over-all time to peak	Antibody units ($\times 10^9$) (Derived for reinjection): At end of first phase of initial rise	Antibody units ($\times 10^9$) (Derived for reinjection): At peak	Rate of formation (k): To end of first phase of initial rise	Rate of formation (k): To peak (derived when initial rise consisted of more than one phase)
1		2	3	4	5	6	7	8
				A. Mean values†				
				Rabbits intact throughout				
1	I	16	2.9	7.3	3.03	3.66	1.43	1.19
	R		2.0	4.6	§	2.42	§	2.09
3	I	16	2.8	7.9	2.59	3.54	1.62	0.99
	R		1.9	4.5	§	4.73	2.15	2.03
5A	I	4	1.9	12.4	9.3	16.9	1.55	0.87
	R		3.4	7.6	§	0.56	§	1.31
5B	I	3	3.0	15.7	1.7	7.9	1.66	0.61
	R		1.8	4.0	§	2.5	§	1.9
6A	I	4	2.3	11.8	8.85	16.8	1.45	0.81
	R		3.1	20.8	2.9	4.6	1.21	0.28
6B	‖	3	See text					
				Rabbits splenectomized when reinjected.				
2	I	13	2.9	7.2	2.1	2.32	1.5	1.26
	R‖		3.0	5.5	§	0.67	§	1.51
4	I	3	3.1	6.0	2.33	3.83	1.68	1.15
	R		2.7	5.4	§	1.13	§	0.99
7A	I	4	2.3	10.8	5.2	12.5	1.35	0.82
	R		4.5	16.1	3.0	9.9	0.67	0.45
7B‖		3	See text					
				Rabbits splenectomized when initially injected.				
8A	I	10	3.8	9.1	0.51	1.3	1.08	0.72
	R	11	2.7	6.5	§	0.67	§	1.47
8B	I	10	3.8	9.1	0.51	1.3	1.08	0.72
	R	13	2.5	5.2	§	1.49	§	1.62
9A	I	4	2.3	13.3	10.0	23.3	1.17	0.74
	R‖		3.2	16.2	§	0.19	§	0.2
9B	I	5	7.6	25.2	3.2	28.7	1.29	0.53
	R		3.2	6.6	1.14	1.28	1.67	1.34
10	I	3	3.0	15.0	9.4	51.5	1.43	0.72
	R		4.1	15.4	11.9	30.0	0.84	0.66
				B. Differences: Initial response (I) minus secondary (R) response‡				
1		16	0.9***	2.7***	0.61	1.24	−0.66*	−0.9***
3		16	0.9***	3.4***	−2.14	−1.19	−0.53***	−1.04***
5A		4	−1.5	4.8	8.74*	16.34**	0.24	−0.44
5B		3	1.2	11.7	−0.8	5.4	−0.24	−1.29
6A		4	−0.8	−9.0	5.95	12.2*	0.24	0.53
2		13	−0.1	1.7***	1.43	1.65*	−0.01	−0.25*
4		3	0.4	0.6	1.2	2.7	0.69***	0.16
7A		4	−2.2*	−5.3	2.2	2.6	0.68	0.37
8A		10–11	1.1	2.6*	−0.16	0.67***	−0.39	0.75***
8B		10–13	1.3*	3.9***	−0.98	−0.19	−0.54	−0.9***
9A		4	0.9	−2.9	9.81*	23.11***	0.97*	0.54
9B		5	4.4*	18.6**	2.06*	27.42*	−0.38	−0.81
10		3	−1.1	−0.4	−2.5	21.5	0.59	0.06

† All mean values are from fitted curves, the operative controls are omitted from the groups.
‡ The significance of the differences are indicated by the following
***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.
§ Rise equivalent to a rise to peak—see next column.
‖ No increase in 2 rabbits at reinjection in series 2, 6B, 7B and 9A.

Rabbit 125 (fig. 1) illustrates the second method of comparing the primary response and two anamnestic responses after reinjection at peak titer and later when antibody was slowly declining. This rabbit had a preimmunization titer of 1:45 and showed a rapid increase in hemolysin to a peak titer of 1:4500 5.5 days after its initial injection. It was reinjected 1.5 days later when the titer was still 1:4500 and 49 days later when the titer had declined to 1:630. Because antibody from the first response was present at the first reinjection, the amount of antibody formed anamnestically could only be conjectured as follows: Since a plateau at peak titer of 6.5 days is abnormally long after the injection of such a small number of red cells (our work, 1950), we assumed (1) that the plateau persisted during the induction period after reinjection and (2) that the rate of decline at −0.14 was delayed for three

TABLE 2.—*Most rapid decline of hemolysin after peak titer. Means and standard errors of apparent half life and number of days decline lasted.*

Immunization		Apparent half life in days	Number of days decline lasted	Number of rabbits in sample	See series
		Single initial and reinjection			
Initial: intact	1.25 ml 1%	6.0±0.3	13.3±0.7	55	1 I*
Reinjection: intact		6.5±0.1	13.1±1.8	12	1 R
Reinjection: splenectomy		11.0±0.5	13.5±1.2	11	2 R
Initial: intact	1 ml 10%	8.6±0.9	18.8±2.0	17	3 I*
Reinjection: intact		6.2±0.4	12.7±1.4	17	3 R
Reinjection: splenectomy		7.0±1.0	9.0±0.6	3	4 R
		Repeated initial and reinjection			
Initial: intact	1.25 ml 1%	10.1±1.0	27.3±2.5	16	6 I*
Reinjection: intact	or	7.6±0.7	21.3±5.0	4	6 R
Reinjection: splenectomy	1 ml 10%	11.2±0.6	30.6±5.5	4	7 R

* Since rabbits of series 2, 4 and 7 were splenectomized at the time of reinjection, data from the initial immunization were combined with the correspondingly treated intact rabbits of series 1, 3 and 6, respectively. Data from the rabbits of series 5 were added to series 6 for the initial immunization and to series 1 or 3 for the reinjection.

days because of the reinjection during which 1500 units/ml were formed at a derived rate of 0.99. A more typical secondary response occurred 49 days later when hemolysin was slowly declining at a rate of −0.028. After an induction period of two days, a peak titer of 1:4000 was reached in two days at one continuous rate of increase ($k=0.8$). At this time, 3545 units/ml were calculated to have been added on the normal baseline of 45 units by assuming the prereinjection titer had continued to decline at a rate of −0.028 and had hence reached 500 units/ml at reinjection peak titer. The actual rate of increase for k was 0.8 and the derived rate of increase was 1.88. After the first reinjection, a rapid primary decline with an apparent half life of five days lasted for nine days and was followed by a more gradual decline with an apparent half life of 30.1 days. After the second reinjection, the apparent half lives of the two declines were 6.9 and 22.3 days, respectively.

The derived amount of antibody formed after reinjection in intact animals is further illustrated in figure 2A. In rabbit 127 (fig. 2A), hemolysin had a preimmunization titer of 1:10, reached a peak of 1:380 and declined to 1:200 at a rate of −0.15 by the ninth day. On this day, the rabbit was reinjected. After an induction period of 2.6 days, an increase in titer to 1:2500 occurred in 1.4 days at a rate of 1.39. Since the titer of the primary response would probably have declined at its original rate to about 120 units in four days, we assumed that 2380 (2500 minus 120) units were added in 1.4 days as a result of the reinjection. When this amount was replotted on the normal baseline of ten units, a derived rate of 2.56 was obtained. In rabbit 129 (fig. 3), the second injection of antigen resulted in no rise in titer. It arrested, however, a rapid disappearance of hemolysin by a plateau of about three days at a titer of 1:5500. Such a plateau, assuming a typical decay rate of −0.126, would require 1700 hemolytic units/ml. This amount equalled a seventh of the initial peak response in the same animal and slightly less than one-half the initial peak response of the group.

The following results may be noted with respect to the initial injection and reinjection in figure 3 and table 1. (1) The induction period was shorter after reinjection. (2) Peak titer was reached sooner. (3) The initial rise after reinjection consisted invariably of one continuous rate of increase (cf. columns 7 and 8 in table 1). The actual rate was lower but the derived rate was higher after reinjection (cf. fig. 1 and 2). (4)

Actual as well as derived peak titers varied markedly even in the same rabbit, but the means were statistically the same. Thus, actual peak titers varied from 1:320 to 1:12,600 after the initial injection and from 1:330 to 1:7500 after reinjection, but differences between the actual or derived (1:1240 in table 1B) peak titers were not statistically significant. (5) The most rapid decline after peak titer was similar in both responses (table 2).

Three of the 16 rabbits had atypically low reinjection peak titers. Two others (125 in fig. 1 and 129 in fig. 3) probably formed considerable antibody, as previously explained. The lack of response in rabbit 139 may be briefly described as follows: The titer was 1:600 on the day of reinjection, decreased to 1:450 on the second day, remained at this level for two more days, rose to 1:530 on the 5th day and decreased to 1:320 by the 8th day. The low anamnestic response in rabbits 74 and 75 was surprising in view of their high initial response. It was not due to the long interval of time (109 days) after the initial injection, because rabbits 73 and 75 gave no more pronounced response when reinjected again with the small amount of antigen 32 days later. Their titers were 1:450 and

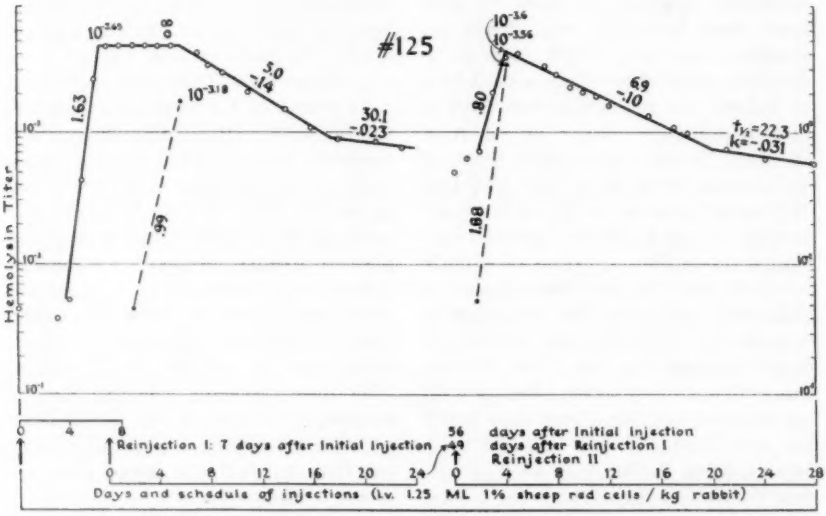


FIG. 1.—Changes in hemolysin titer in intact rabbit 125 from series 1 after an initial injection of 1.25 ml of 1% red cells/kg and after two reinjections 7 and 56 days later.

Arrows at the bottom of the figure indicate the times of injecting the sheep red cells. The same relative amount was always injected into a given rabbit and was always given intravenously. Peak titers are from the fitted curves. The values for k showing rates of increase or decline of antibody lie adjacent to the lines from which they were calculated. The figure above the $-k$ value represents the apparent half life of antibody in days. Solid lines are fitted from the actual titers. Broken lines are drawn to give a single rate of increase from preimmunization titer to peak titer and for the secondary response necessitated subtracting the existing titer of the primary response.

Note the single rate of increase to peak titer after the first injection and the prolonged plateau at peak titer. The actual rate of increase after the second reinjection (0.8) was not higher than the initial actual rise (1.63), but the derived rate (1.81) was higher when realigned on the preimmunization baseline.

1:130 at the time of the second reinjection and rose to 1:1400 and 1:260 six days later.

Series 2 (13 rabbits splenectomized when reinjected).—The even-numbered rabbits between 126 and 148 and 92 were injected initially and a second time as were those in series 1. On the day of the reinjection, they were splenectomized. Titers at the time of reinjection ranged from 1:40 in rabbit 148 to 1:1800 in rabbit 134.

Figure 4 illustrates the greatly reduced antibody response due to splenectomy. Peak titer was only higher than the initial peak in one rabbit (140). In two rabbits the titer steadily decreased after reinjection (from 1:560 to 1:350 in five days in 130 and from 1:750 to 1:340 in three days in 138). On the other hand, peak titer was reached sooner (5.5 days), but not as much sooner (4.6 days) as in the intact rabbits. The same statement may be made with respect to the rate of accumulation. The induction period was not significantly shorter (table 1A and B).

The difference between the anamnestic response in the same rabbit when intact and when splenectomized is shown in figure 2. Rabbit 127 was given one reinjection while intact, nine days after its initial injection, and a second reinjection on the day it was splenectomized, 62 days later. After splenectomy, less antibody accumulated at a lower rate, both with respect to the actual (0.29 vs. 1.39) and derived (0.54 vs. 2.56) values for k. Rabbit 128 (fig. 2B) was reinjected and splenectomized on the ninth day. It formed less antibody after splenectomy than initially. Rabbits 131 and 132 showed the same effects of splenectomy (fig. 5). The low

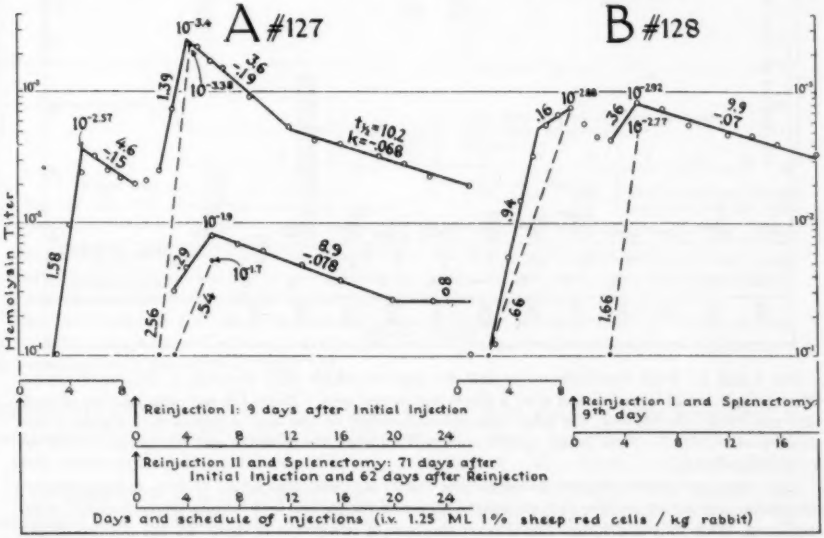

A #127
B #128
Hemolysin Titer
Reinjection I: 9 days after Initial Injection
Reinjection I and Splenectomy 9th day
Reinjection II and Splenectomy: 71 days after Initial Injection and 62 days after Reinjection
Days and schedule of injections (i.v. 1.25 ML 1% sheep red cells / kg rabbit)

FIG. 2A and B.—Changes in hemolysin titer in rabbit 127 from series 1 and in rabbit 128 from series 2 after an initial injection and after one or two reinjections with 1.25 ml 1% red cells/kg. Rabbit 127 was intact through the intial injection and reinjection and was splenectomized at the second reinjection. Rabbit 128 was splenectomized at the first reinjection. See figure 1 for other explanations.

Note the marked anamnestic response nine days after the initial injection in intact rabbit 127. Little antibody was formed after splenectomy at nine days in rabbit 128 or at 71 days in rabbit 127.

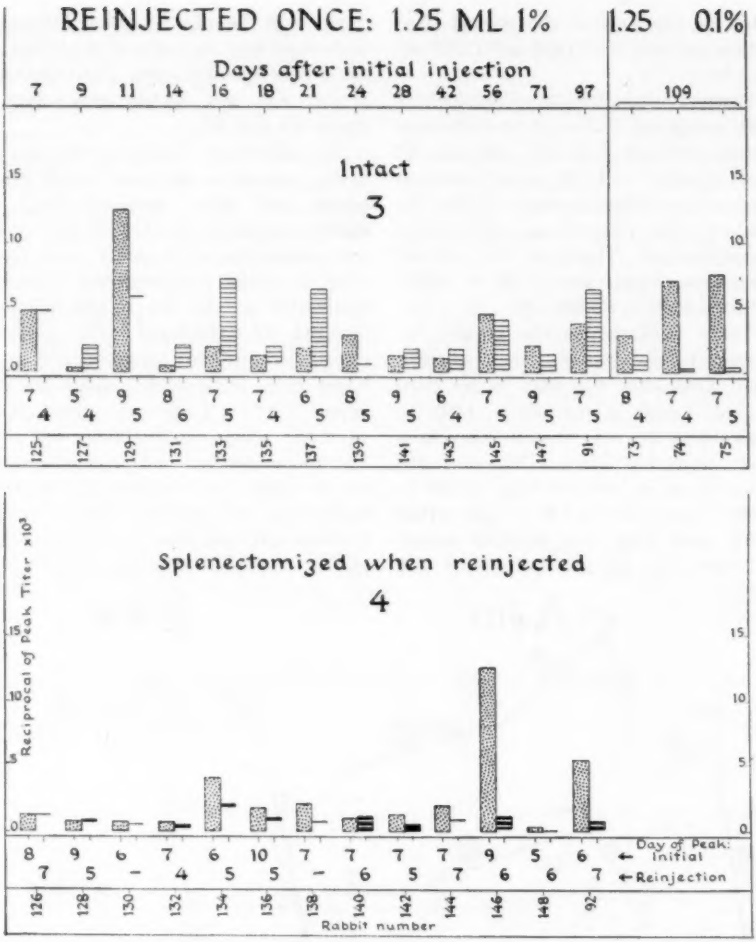


FIG. 3 and 4.—Peak hemolysin titers and the days on which they occurred in 13 pairs of rabbits after a single initial injection and after a single reinjection with 1.25 ml 1% red cells/kg. One of each pair was intact throughout: the other was splenectomized on the day of reinjection. Figure 3 also shows similar data for three intact rabbits from series 1 after the injection and reinjection of 1.25 ml 0.1% red cells/kg.

Peak titers and times are from fitted curves. Initial injection into intact rabbits, dotted column; reinjection into intact rabbits, thin striped column; and reinjection into rabbits splenectomized when reinjected, thick striped column. The titer at the time of reinjection is shown by the position of the bottom of the striped column.

Note that (1) the peak after the reinjection was reached sooner than after the initial injection in both intact and splenectomized rabbits, (2) it varied within the same range as the initial injection in intact rabbits, and (3) it was in general higher in the intact than in the splenectomized rabbits.

response of splenectomized rabbits to a reinjection is in accord with the response of splenectomized rabbits to an initial injection (our paper, 1950, and the regularly dotted columns in figure 14).

rabbits and three operative controls (testis or appendix removed at the initial injection) were injected twice with the large number of sheep cells within 45 to 120 days. In addition, two

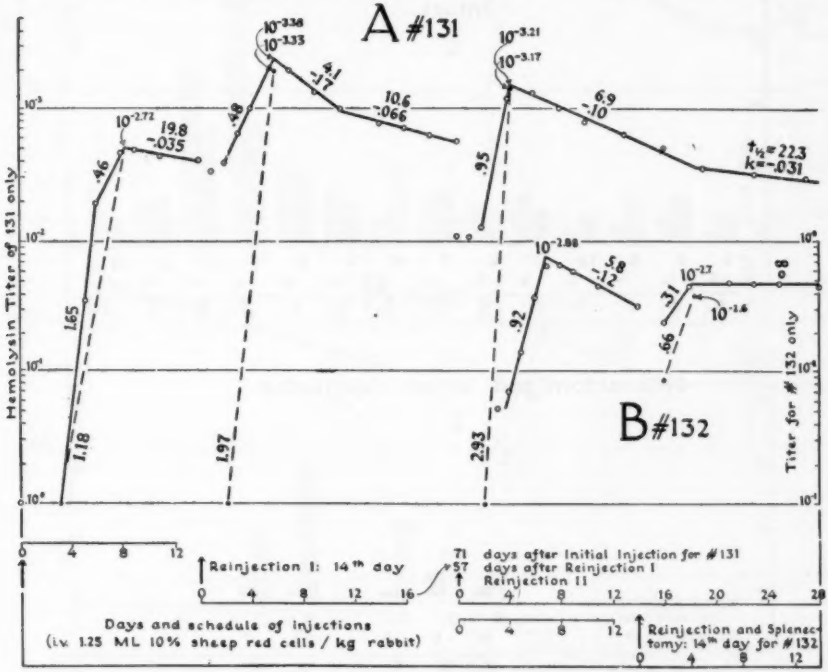

A #131
B #132
Hemolysin Titer of 131 only
Titer for #132 only
Reinjection I: 14th day
71 days after Initial Injection for #131
57 days after Reinjection I
Reinjection II
Days and schedule of injections
(i.v. 1.25 ML 10% sheep red cells / kg rabbit)
Reinjection and Splenectomy: 14th day for #132

FIG. 5A and B.—Changes in hemolysin titer in rabbit 131 from series 1 and in rabbit 132 from series 2 after an initial injection and one or two reinjections. Rabbit 131 remained intact throughout: rabbit 132 was splenectomized when reinjected. See figure 1 for other explanations.

Note two changes in the rate of increase during the initial rise in intact rabbit 131 (1.65 and 0.46) and the high single derived rates of increase (1.93 and 2.93) after the two reinjections. Splenectomy depressed antibody formation in rabbit 132.

Experiment 2 (series 3 and 4). A single reinjection of 1 ml of 10% sheep red cells into intact and splenectomized rabbits

The results in this experiment corroborated and extended the results of experiment 1. Eight times as much antigen was injected 45 to 223 days after the primary injection (fig. 6 through 8).

Series 3 (12 rabbits).—Nine intact

of the nine rabbits were reinjected a second time, 176 days after the first reinjection. The titer in these rabbits at the time of reinjection varied between 1:40 in rabbit 108 to 1:350 in rabbit 43.

The similarity between this series and series 1 may be seen by comparing figure 6 with 3 and series 3 with 1 in table 1A and B. Initial peak titers

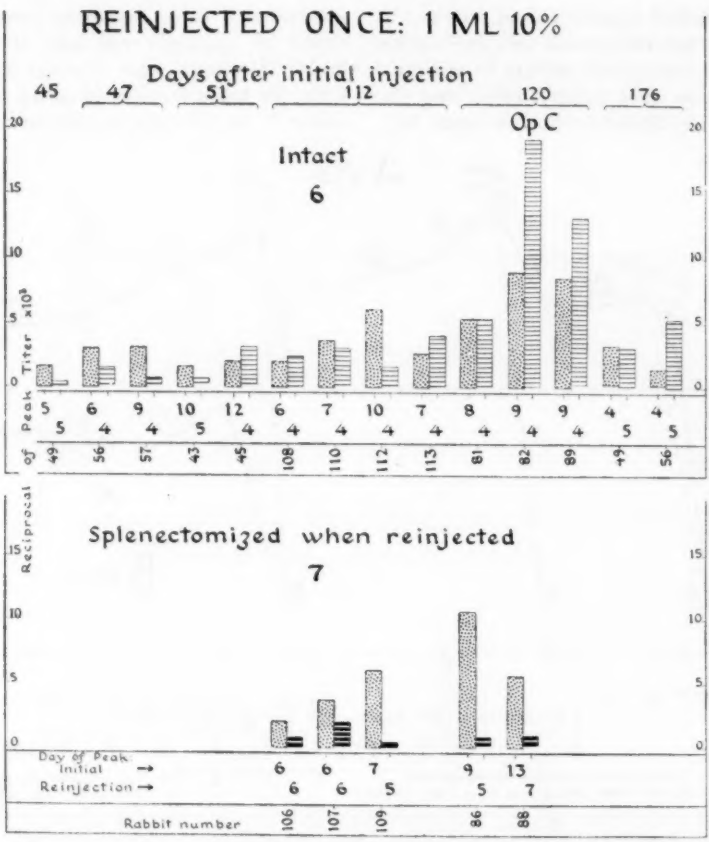


FIG. 6 and 7.—Peak hemolysin titers and the days on which they occurred in two sets of rabbits after a single initial injection and after a single reinjection with 1 ml 10% red cells/kg. One set (series 4) was splenectomized on the day of reinjection: the other set (series 3) was not. Five of the rabbits (Op C) had a testis, kidney or appendix removed when initially injected. See figures 3 and 4 for other explanations.

Note that (1) the anamnestic response in the intact and splenectomized rabbits was similar to that noted in figures 3 and 4; (2) it was high in the operated nonsplenectomized rabbits; and (3) it was low in splenectomized rabbits including the operative controls (86 and 88).

varied from 1:1600 to 1:8900 and those of the reinjection from 1:400 to 1:19,000. Those of the reinjection were only higher in four of the nine intact rabbits and in two of the three operative controls. The apparently more pronounced response at 120 days was probably due to the greater reactivity of the operated rabbits as already described by us (1950). In any case, the responses in rabbits 49 and 56 were no higher than in the rest of the rabbits although 176 days had elapsed between the two reinjections and 223 days since the initial injection. One continuous rate of increase to peak titer occurred in all

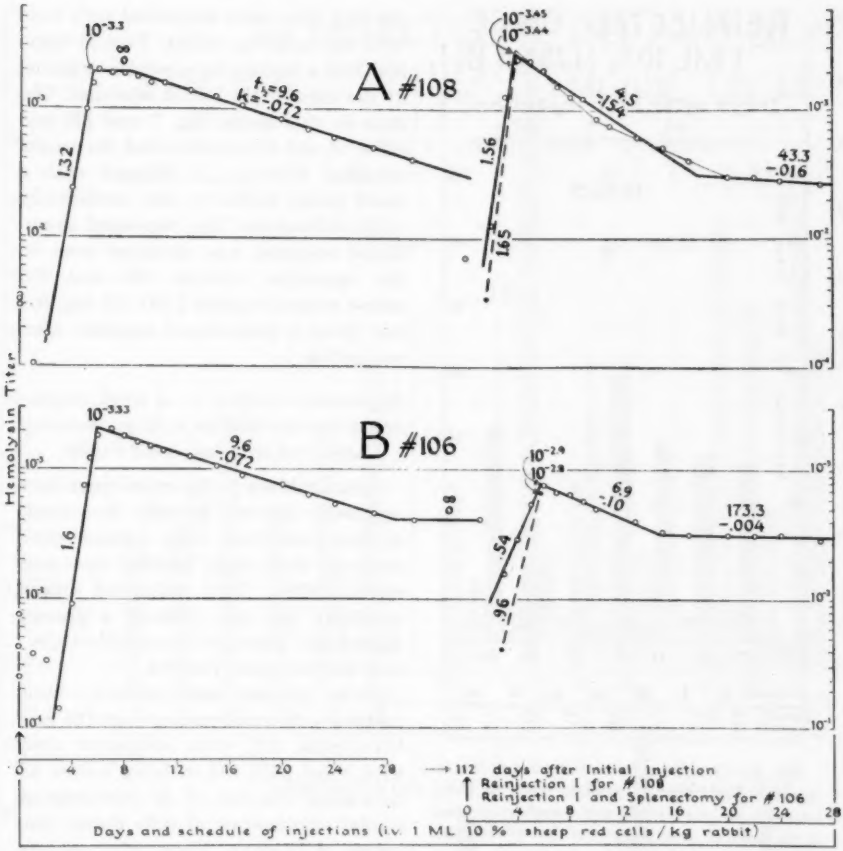


FIG. 8A and B.—Changes in hemolysin titer in intact rabbit 108 from series 3 and in rabbit 106 from series 4 (splenectomized when reinjected) after an initial injection of 1 ml 10% red cells/kg and a reinjection 112 days later. See figure 1 for other explanations.

Note that the derived rate of increase (1.65) after reinjection approached the actual rate (1.56) in rabbit 108 because little antibody from the primary response remained at 112 days.

except two rabbits after reinjection. The initial rise in these two consisted of two phases. In rabbit 43, a rate of 0.35 continued for 1.8 days and was followed by a rate of 0.001 for three days. In rabbit 45, a rate of 0.89 continued for 3.4 days and was followed by a rate of 0.2 for five days. When reinjection was postponed to 112 days (fig. 8A), the actual rate of increase (1.56) almost approached the derived rate of increase (2.01) because little antibody from the primary response remained. Parenthetically, it may be noted that rates could occasionally be calculated in two or more ways. As seen in figure 8A, antibody could be assumed to decline at a rate of -0.154 for 17 days or by using the alternate lines seen in the figure at a rate of -0.2 for six days and -0.1 for

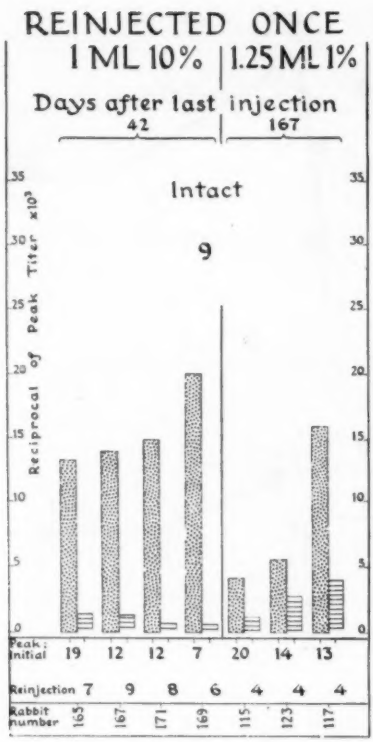


FIG. 9.—Data from series 5 similar to that in figure 3. These rabbits received repeated intravenous injections initially and a single reinjection of the large or intermediate amount of red cells. See figures 3 and 4 for other explanations.

Note the partially refractory period in the rabbits reinjected at 42 days and recovery at 167 days.

13 days. In such cases, we preferred to delineate the antibody curves with as few rate changes as possible.

Series 4 (five splenectomized rabbits).—These rabbits were splenectomized on the day they were reinjected with 1 ml 10% red cells/kg rabbit. Two of them had had a kidney or appendix removed at the time of the initial injection. The data in this series (fig. 7 and 8B and table 1A and B) corroborated the results obtained in series 2, although such a small group failed to give statistically valid differences. The depressed anamnestic response was obtained even in the operative controls (86 and 88) whose mates in series 3 (81, 82 and 89) had given a pronounced response after reinjection.

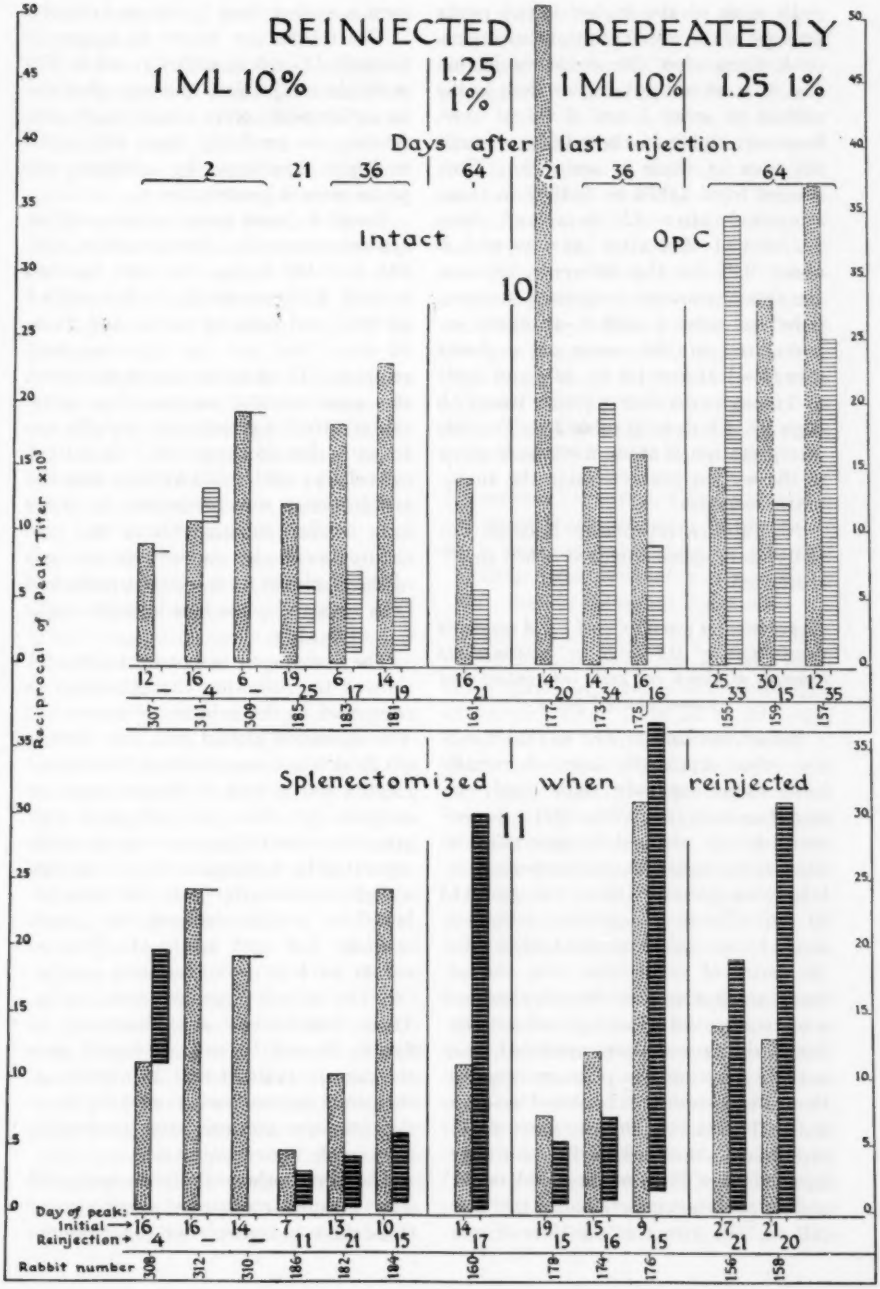
Experiment 3 (series 5). A single reinjection of the intermediate or large amount of sheep red cells into intact rabbits

Intact rabbits in this experiment were repeatedly injected initially. As a result of this procedure, they formed more antibody than singly injected ones (our work, 1951). This enhanced initial reactivity did not produce a greater anamnestic response to a single reinjection than in series 1 and 3.

Series 5 (seven intact rabbits).—Four rabbits (odd numbers between 165 and 171 = series 5A) were reinjected once with 1 ml 10% red cells/kg rabbit 42 days after the last of 34 injections of similar suspensions of cells during the course of 73 days. Three other rabbits (115, 117 and 123 = series 5B) were reinjected with the intermediate amount of red cells 167 days after the last of ten injections of this amount during the course of 26 days. At the time of reinjection, titers varied from 1:260 to 1:450 in the first four rabbits and from 1:170 to 1:380 in the last three.

FIG. 10 and 11.—Data from series 6 and 7 similar to that in figures 3 and 4. These rabbits received repeated intravenous injections initially and the same number of reinjections. The last five pairs plus one extra (Op C) had a testis removed on the day of the initial injection of red cells. Series 7 was splenectomized on the day of the first reinjection. See figures 3 and 4 for other explanations.

Note that the anamnestic response was less intense than the primary one in 13 of the 14 intact or splenectomized rabbits and in six of 12 of the operative controls. In general, the peak was reached later.


REINJECTED REPEATEDLY
1 ML 10%
1.25 1%
1 ML 10%
1.25 1%
Days after last injection
2
21
36
64
21
36
64
Intact
Op C
10
Reciprocal of Peak Titer x10³
Splenectomized when reinjected
11
Day of peak:
Initial →
Reinjection →
Rabbit number

In spite of the higher initial peaks because of the initial multiple injections, peak titers after the single reinjection (fig. 9) were not any higher than in the rabbits of series 1 and 3. They were, however, higher in the rabbits of series 5B than in those of series 5A. They ranged from 1:670 to 1:1000 in those reinjected after 42 days and from 1:1260 to 1:4000 after 167 days with P about 0.05 for the difference between the two anamnestic responses. As compared to series 1 and 3, antibody accumulated in this series at a slower rate ($k = 1.31$ and 1.9 vs. 2.03 and 2.09) and in series 5A over a longer time (7.6 days vs. 4.5 days in table 2A). Possible interpretation of these findings is given in the section characterizing the anamnestic response.

No rabbits, repeatedly injected initially, were splenectomized when singly reinjected.

Experiment 4 (series 6 and 7). A multiple reinjection of the large or intermediate amount of sheep red cells into intact and splenectomized pairs

Splenectomized as well as intact rabbits when repeatedly injected initially form more antibody than singly injected animals (our work, 1951). Experiment 4 was planned to ascertain the anamnestic response after repeated reinjections (rabbits 155 to 161 and 173 to 186). The even-numbered rabbits in series 7 were splenectomized on the day the series of reinjections was started. Both mates of some of the pairs had had a testis removed when injected initially. Such rabbits sometimes produced more antibody during the primary response than intact rabbits (cf. dotted columns in figs. 10 and 11). The two parts of this experiment consisted of 19 rabbits reinjected after 21 to 64 days (6A or 7A) and six rabbits reinjected after two days (6B or 7B). Titers at the time of reinjection varied from 1:100 to 1:24,000.

The results are shown in figures 10 through 13 and in tables 1 and 2. The multiple reinjections did not give rise to earlier peaks or to a more rapid production of antibody than the initial multiple injections. In addition, the peaks were in general lower.

Series 6 (seven intact rabbits and six operative controls).—Three rabbits (181, 183 and 185 in fig. 10) were injected initially 17 times during 34 days with 1 ml 10% red cells/kg rabbit and 21 to 36 days after the last injection were reinjected 17 times during 34 days with the same relative amount. One other rabbit (161) was injected initially ten times during 26 days with 1.25 ml 1% red cells/kg rabbit and 64 days after the last injection was reinjected ten times in a similar manner. These two procedures were also carried out on each of three rabbits from which a testis had been removed when first injected (right half of fig. 10).

The anamnestic response in series 6A showed the following characteristics as compared to the primary response. (1) The induction period was not shorter. (2) Peak titers were lower in four intact rabbits and in four of the six operative controls. (3) They were reached later after two rates of increase—occasionally separated by a plateau. In two rabbits, a slight decline after peak titer was followed by a slight increase for a week (rabbits 175 and 183). (4) Derived values for k to peak titer were smaller. (5) The primary decline was similar. These conclusions are illustrated in figures 10 and 12 and are based upon the data in tables 1 and 2. In addition, the peaks on the average were higher in the operative controls after reinjection than in the intact animals.

The three other rabbits in series 6B were injected repeatedly every two or three days as controls for three rabbits

splenectomized on the 14th day (see next section).

Series 7 (12 splenectomized rabbits of which five had had a testis removed when initially injected).—Nine rabbits (series 7A in table 1 and fig. 11) were treated by one of the two procedures described for series 6A and only differed from them by being splenectomized on the first day that the second series of reinjections was started. Titers ranged from 1:100 (rabbit 158) to 1:1600 (rabbit 176) at the beginning of the second series of injections.

The induction period was significantly longer in intact animals in contrast to the singly injected series. The relatively high peak titer after reinjection was in marked contrast to the depressing effect of splenectomy on anamnesis in the first experiments (cf. fig. 11 with fig. 4 and 7). This observation is in agreement with our earlier finding that splenectomy increased peak titer after initial immunization with a series of injections. A typical sequence is shown in figure 13.

In view of these results, three more rabbits (308, 310 and 312) were splenectomized during the course of continuous injections with 1 ml 10% red cells/kg (6B in fig. 11). They were given 17 injections, daily or every other day during the course of 28 days and were splenectomized two days after the eighth injection or 14 days after the first injection. Through the 14th day, the titer increased as in rabbit 181 (fig. 12) or as in rabbit 182 (fig. 13). On the 14th day, it had reached 1:11,000 in rabbit 308, 1:19,000 in rabbit 310 and 1:24,000 in rabbit 312. Titers were so high in these rabbits at the time of splenectomy and nonsplenic sources were already adding such a large amount of antibody that it was difficult to assess the amount of antibody formed after splenectomy during continued antigenic stimulation. It is interesting, however, that there seemed to be no change in the type of curve which could be ascribed to splenectomy. Thus, antibody increased from 11,000 to 19,500 units/ml in four days in rabbit 308 at a continuing presplenectomy rate of 0.15 and failed to rise in the other two (fig. 11). This result is in line with our earlier assumption (1951) that the spleen at this time has ceased to form antibody. Afterwards a plateau or slow decline lasted until one day before to six days after the last injection and a rapid decline then set in in all three rabbits with an apparent half life between 4.6 and 6.9 days. The intact rabbits behaved similarly (307, 309 and 311 in fig. 10).

Experiment 5 (series 8, 9 and 10). Single and multiple reinjections of the intermediate or large amount of sheep red cells into rabbits splenectomized at or near the time of the initial injection

The compensatory activity of nonsplenic sites in anamnesis was studied in three series of rabbits splenectomized at varying times from 22 to 193 days prior to the reinjection. In series 8, there was only one initial injection and one reinjection. In series 9, rabbits were injected repeatedly at first and were given a single reinjection. In series 10, the same series of multiple injections was given twice. The experiment is illustrated by figures 14 through 17 and additional data may be found in table 1.

Series 8 (20 rabbits splenectomized three weeks or more prior to the reinjection).—These rabbits, variously numbered from 36 to 105, were splenectomized from 28 days before (95 to 97) to eight days after (53 to 55, 104 and 105) an initial injection of 1 ml 10% red cells. They were reinjected from 22 to 112 days later with the same number of red cells/kg. Their titers at the time

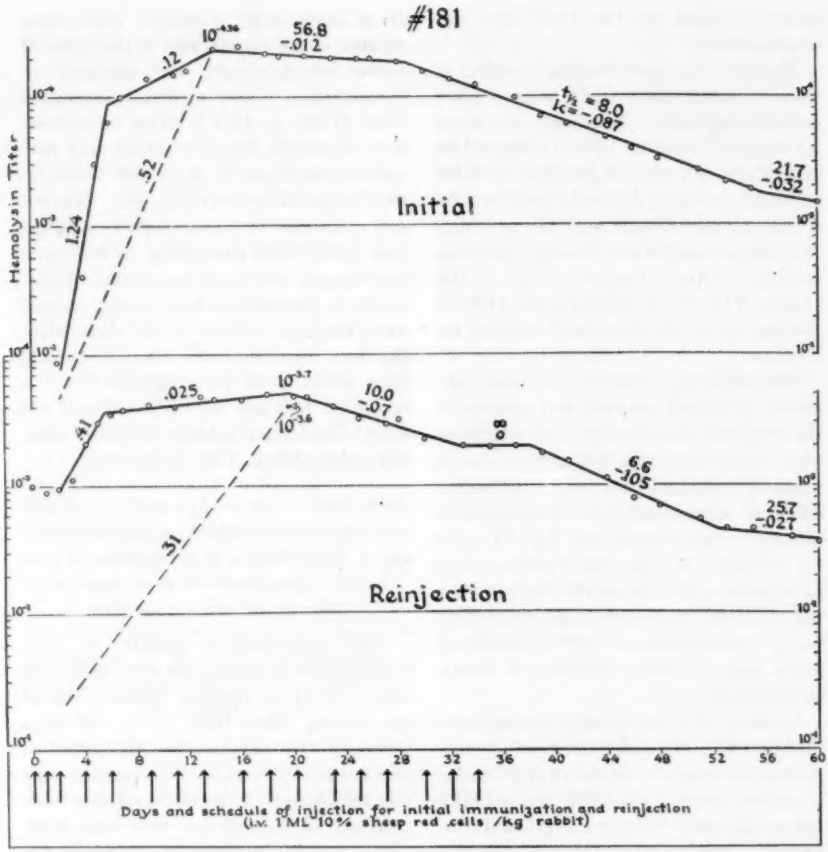

#181
Hemolysin Titer
Initial
Reinjection
Days and schedule of injection for initial immunization and reinjection
(i.v. 1 ML 10% sheep red cells /kg rabbit)

FIG. 12.—Changes in hemolysin titer in intact rabbit 181 from series 6 which received 17 initial injections of 1 ml 10% red cells/kg and, 36 days after the last injection, a similar number of reinjections. See figure 1 for other explanations.

Note the two changes in rate of increase during the initial rise of both the primary and secondary response. Peak titer was not reached until two weeks or more.

of reinjection varied from 1:10 in rabbit 105 to 1:700 in rabbit 53.

Initial peak titers are represented in figure 14 by the irregularly dotted column when splenectomy was performed six to eight days after the initial injection (50 to 55, 100, 101, 104 and 105) and by the regularly dotted column when splenectomy was performed before or through five days after the initial injection. This device was used because hemolysins had had time to be produced in rabbits splenectomized six to eight days after they had received antigen but not in those splenectomized earlier. The mean peak titer of the primary response for the first group was 1:3067 $\pm$1:414 as compared to 1:1016$\pm$1:267 for the second group.

Peak titers after the reinjection were

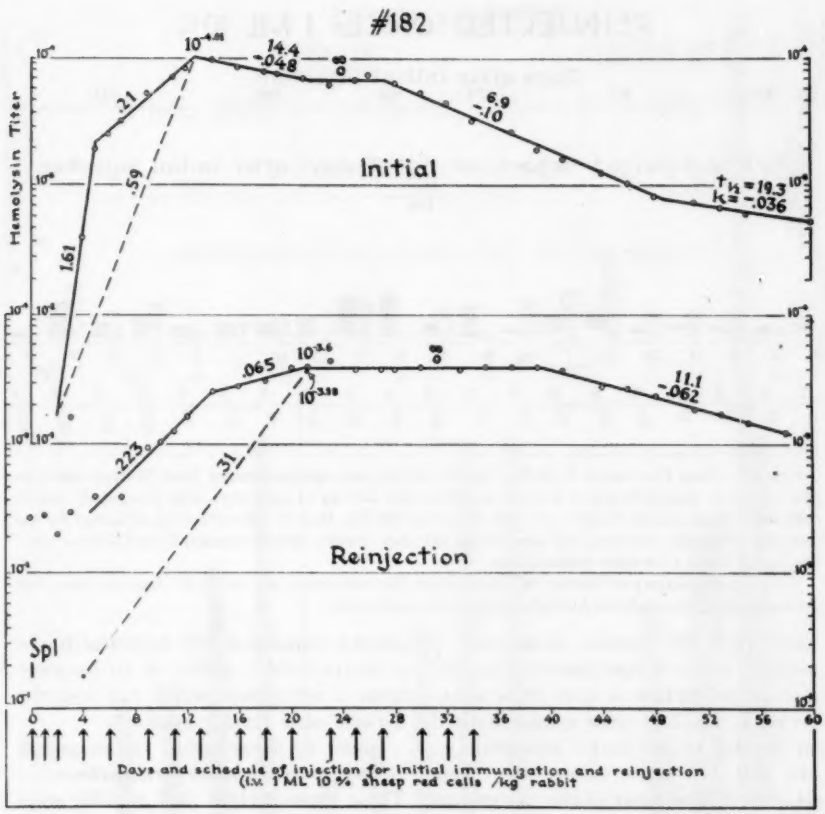


FIG. 13.—Changes in hemolysin titer in rabbit 182 from series 7 injected and reinjected as was rabbit 181 in figure 12. This rabbit was splenectomized on the first day of the series of reinjections. See figure 1 for other explanations.

in general lower when the reinjection was made 47 days or less (series 8A) after the initial injection than when made later (series 8B). Before 50 days, they varied from 1:420 in rabbit 55 to 1:2000 in rabbit 53 (mean = 1:1030 ± 1:207) and after 50 days, aside from 105, which was unreactive, they varied from 1:900 in rabbit 98 to 1:3300 in rabbit 83 (mean = 1:1845 ± 1:256). This finding seems to indicate that some compensatory action had taken place after a month and a half.

Rabbits 36, 39 and 47 were reinjected a second time 48 days after the first reinjection and rabbit 36 a third time 55 days after the second reinjection. Peak titers were reached within the same length of time as after the first reinjection but were lower. They were, in order, 1:500, 1:2200, 1:90 and 1:350.

Series 9 (nine rabbits splenectomized either 16 or 27.5 weeks prior to reinjection).—Groups of four (even numbers between 166 and 172 = series 9A) and five (even numbers between 116 and

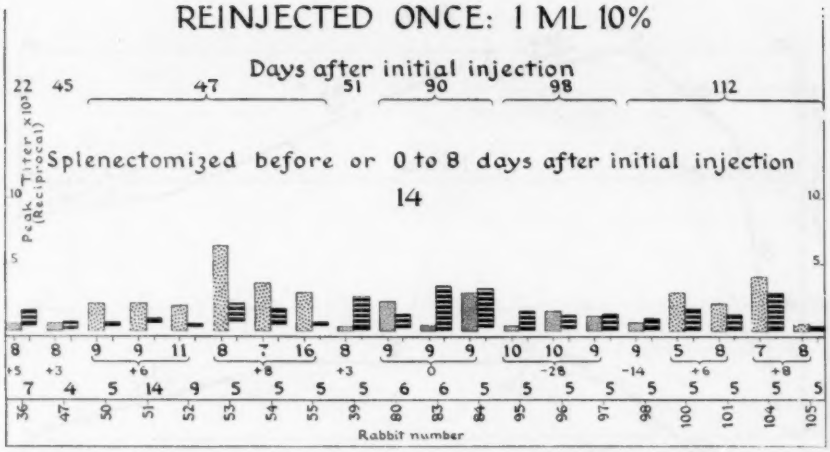


FIG. 14.—Data from series 8. Half of the 20 rabbits were splenectomized from 28 days before to five days after (regularly dotted column) and the other half six to eight days after (irregularly dotted column) a single initial injection of 1 ml 10% red cells/kg. Day of splenectomy is indicated by the numbers below the brackets. All were reinjected once (heavy striped column) 22 to 112 days later. See figures 3 and 4 for other explanations.

Note a compensatory action for the spleen when the reinjection was made 51 days or more after the initial injection as shown by higher peaks after reinjection.

124 = series 9B) rabbits were given a multiple series of injections initially and a single reinjection as were those in experiment 3. They were splenectomized on the day of the initial injection, i.e., 115 and 193 days before they were reinjected. The titers of the two groups when reinjected varied from 1:500 to 1:1260 and 1:75 to 1:630, respectively.

In spite of the high primary response in these splenectomized rabbits, the anamnestic response to a single reinjection was within the range encountered in series 9 (cf. fig. 15 with 14). In rabbit 172, there was a plateau for two weeks. In rabbits 168 and 170, the titer, after a preliminary decrease, increased slightly but not to as high a titer as at the time of the test reinjection. In the rabbits in series 9B, the mean peak titer was higher after the longer interval (difference = 720 ± 180 units/ml with $P = 0.005$). Whether the greater response at 167 days was due to a compensatory action or to recovery from a refractory period can only be decided after further work.

Series 10 (three rabbits splenectomized 70 days prior to a series of reinjections).—These three rabbits (187 to 189) were splenectomized on the day an initial immunization schedule of 17 injections was begun similar to that described for the majority of the rabbits in experiment 4. Seventy days after splenectomy or 36 days after the last of the initial immunization injections, they were reinjected similarly. Titers ranged from 1:1700 to 1:6700 at the time of reinjection.

Peak titers in these rabbits were not as high during the second series of injections as at first although they were reached in the same length of time (table 1 and fig. 16). The anamnestic response, however, was somewhat more pronounced than in intact rabbits 181,

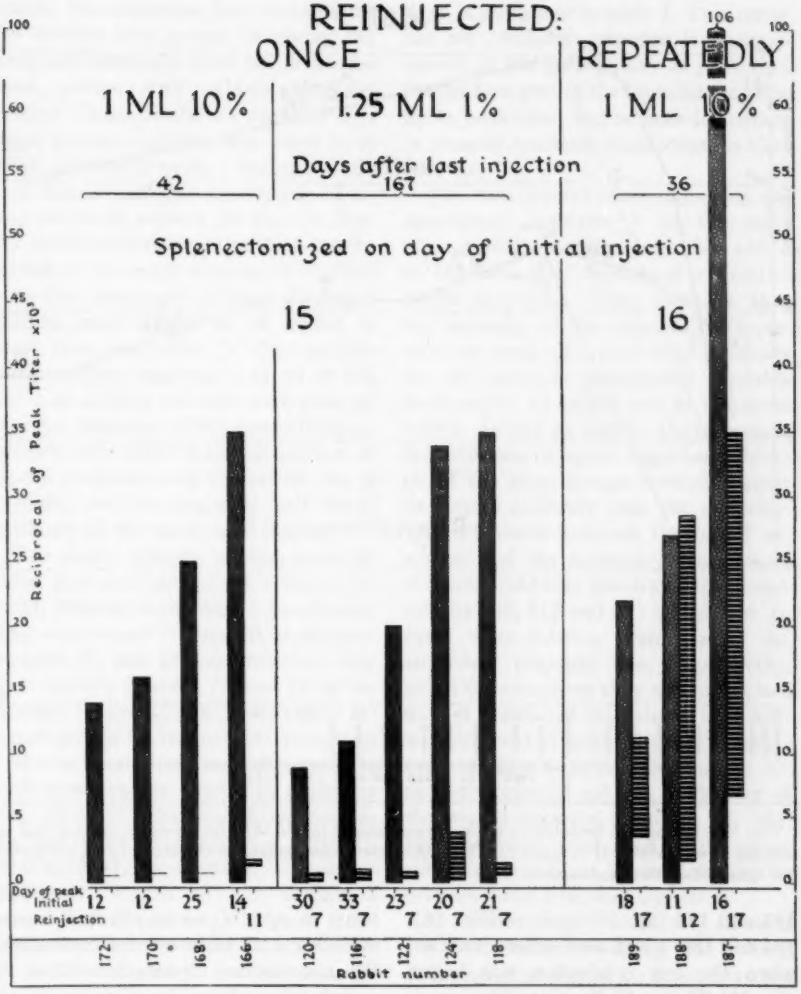


FIG. 15.—Data from series 9. Nine rabbits were splenectomized on the first day of a series of initial injections and were reinjected once, 42 (first four) or 167 (last five) days after the last injection of the initial series. Injections into these rabbits were carried out at the same time as those in figure 9. See figures 3 and 4 for other explanations.

Note that the anamnestic response was more marked after the longer interval.

FIG. 16.—Data from series 10. These three rabbits were splenectomized on the first day of a series of initial injections and were reinjected with a similar series 36 days after the last one of the initial series. Injections into these rabbits were carried out at the same time as those in figures 10 and 11. See figures 3 and 4 for other explanations.

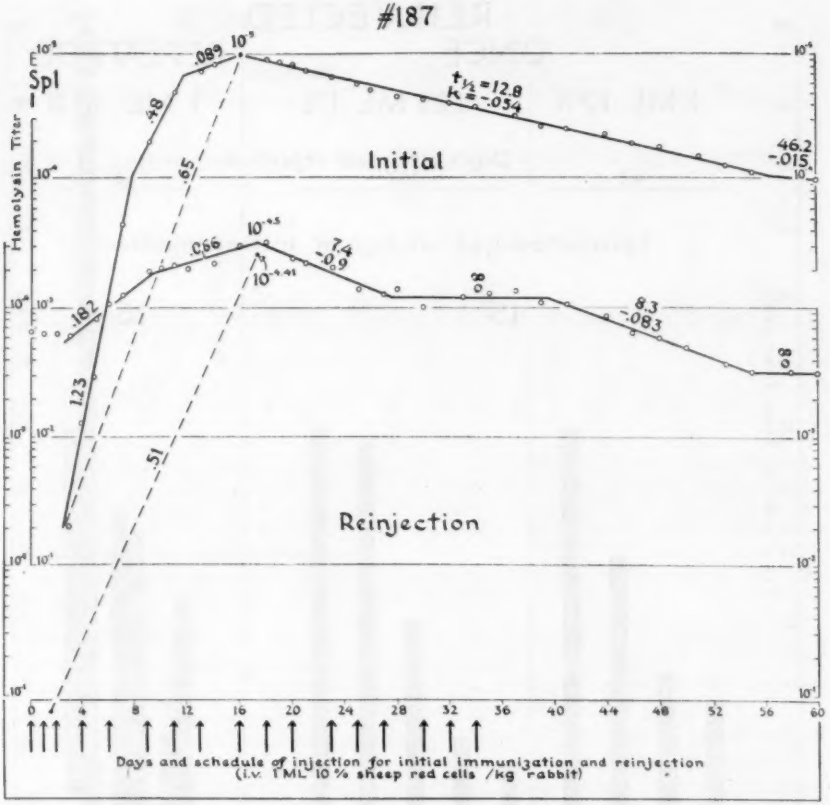


FIG. 17.—Changes in hemolysin titer in splenectomized rabbit 187 from series 10, injected and reinjected as was rabbit 181 in figure 12. This rabbit was splenectomized on the day it was first injected. See figure 1 for other explanations.

183 and 185 (fig. 10) or in rabbits 182, 184 and 186, which were splenectomized when the test reinjection was begun (fig. 11). Figure 17 illustrates the course of the primary and anamnestic responses in this group. The figure is also of interest because this rabbit elicited the highest peaks (1:106,000 and 1:35,000) so far obtained for either response in our work.

Characterization of the secondary response

In describing the primary and secondary responses in the ten preceding series of rabbits, we emphasized serum antibody and experimental conditions. We also utilized Student's method of paired comparison for statistical analysis. This pairing of the responses in the same rabbit gave little advantage because (1) the peak titers for the two responses in the same animal were not significantly correlated although the mean titer was not significantly different, and (2) antibody production was statistically the same in each similarly treated group except during the refractory period after multiple initial immun-

ization. We, therefore, have recombined the rabbits into groups involving the same treatment and have supplemented some groups with rabbits similarly treated. Thus, the initial response to a single antigen injection was tested in 44 intact rabbits in series 1 through 4 and to a repeated antigen injection in 15 intact rabbits in series 5, 6A and 7A. Similar initial treatments of rabbits, not included in the series because the rabbits were not reinjected, allowed 23 and 6 rabbits, respectively, to be added to these two categories. In this realignment, rabbits receiving 1.25 ml of 1% and 1 ml of 10% red cells were grouped together since we (1950) found their responses only differed in the number of phases characterizing the initial rise of antibody and the apparent half life of antibody of the most rapid decline.

The singly injected rabbits were divided into four groups (A through D) which differed with respect to whether they were intact (A and B) or splenectomized (C and D) and whether they were initially injected (A and D) or reinjected (B and C). Four repeatedly injected groups (E through H) differed in a like manner. Frequency distributions with mean values were then made for each group of peak titer (fitted and, in the case of the test reinjection, derived), total antibody for 30 days, constant of increment, length of the induction period and the interval to peak titer. These data are graphed in figures 18, 19 or 20 and differences between the means of various groups with their statistical significance are given in table 3. The means of four of these five values are essentially similar to the means for the individual series of rabbits in columns 3, 4, 6 and 8 of table 1. The few discrepancies in absolute values between the two sets of data (table 1A vs fig. 18 through 20) and in the statistical validity of the differences (tables 1B and 3) are minor. Total antibody for 30 days is not given in table 1. This measure for antibody response is approximately of the same utility as peak titer for the first part of the immune response up to peak titer, but is more important in gauging antibody production as time goes on.

The data involve observations on the anamnestic response 7 to 176 days after a single antigen injection and 2 to 167 days after the last of a series of initial injections. They indicate that the intensity of the response, as measured by peak titer and total antibody for 30 days, is remarkably variable from rabbit to rabbit and in the same rabbit. As may be seen by the frequency distributions in figure 18, a few rabbits in all the large groups formed a great deal more antibody than the majority. The difference between the initial response and the secondary response in the same rabbit is illustrated by intact rabbits 108, 112 and 113 in figure 6. In these three rabbits, peak titer of the secondary response was, respectively, about the same, less than and more than that of the initial response. This result was obtained in spite of identical treatment of the three rabbits. In the repeatedly injected rabbits, peak titer of the second response in rabbit 181 (fig. 10) was lower than that of the other three intact rabbits in series 6. Yet its primary peak titer was highest.

Aside from such variability, all data are consistent in indicating that a sharp differentiation should be made between the secondary response elicited by a single antigen injection and that elicited by repeated injections. This same distinction was made previously for the primary response (our work, 1951). The main point of departure is the relative greater importance of the spleen over nonsplenic antibody-forming sources after single injections in both primary and secondary responses. Thus, when peak titers after reinjection of compar-

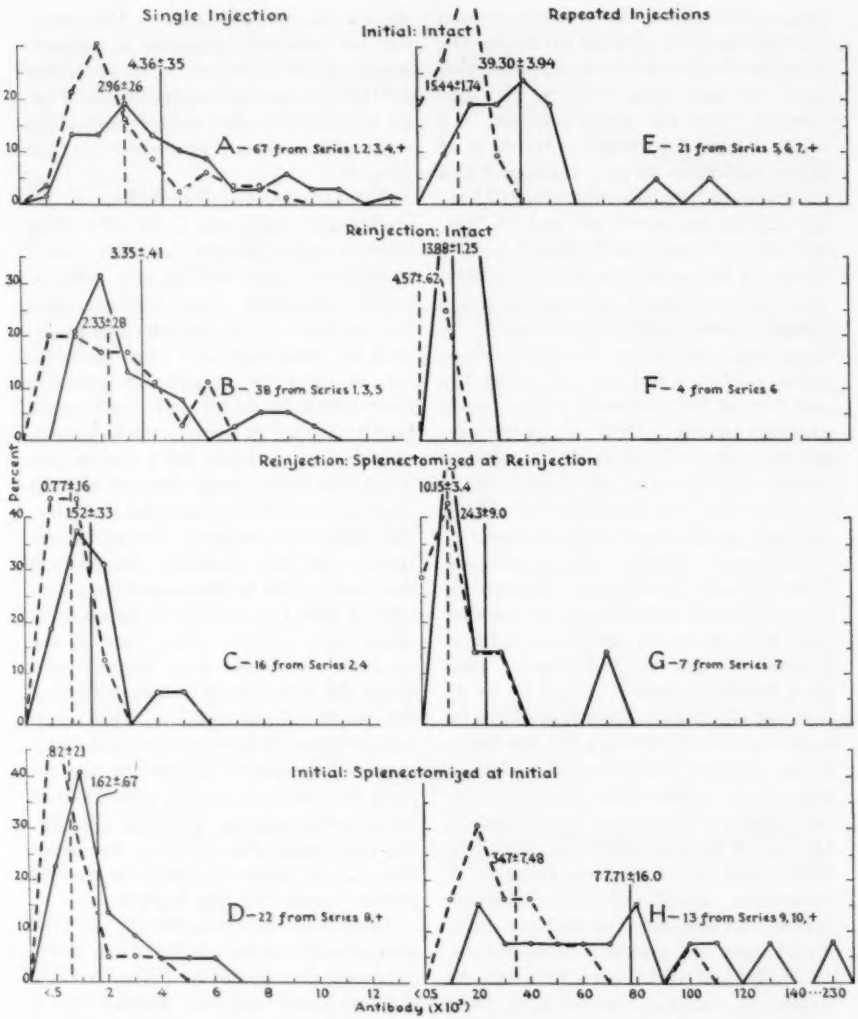


FIG. 18.—Percentage frequency distributions for fitted peak titer (dash lines) and for total antibody for 30 days (solid lines) in eight groups of rabbits (A through H) treated differently with respect to a single or repeated initial injections and reinjections and splenectomy. The derived peak titer was used after reinjection. Means and standard errors are also included. Additional rabbits indicated by + in the figures were added to some groups, whereas the operative controls in all experiments and the reinjections in experiment 5 were omitted. For the statistical significance of differences between various groups, see table 3.

Note that the curves for peak titer resemble in general the curves for total antibody in any particular group although the means are obviously smaller.

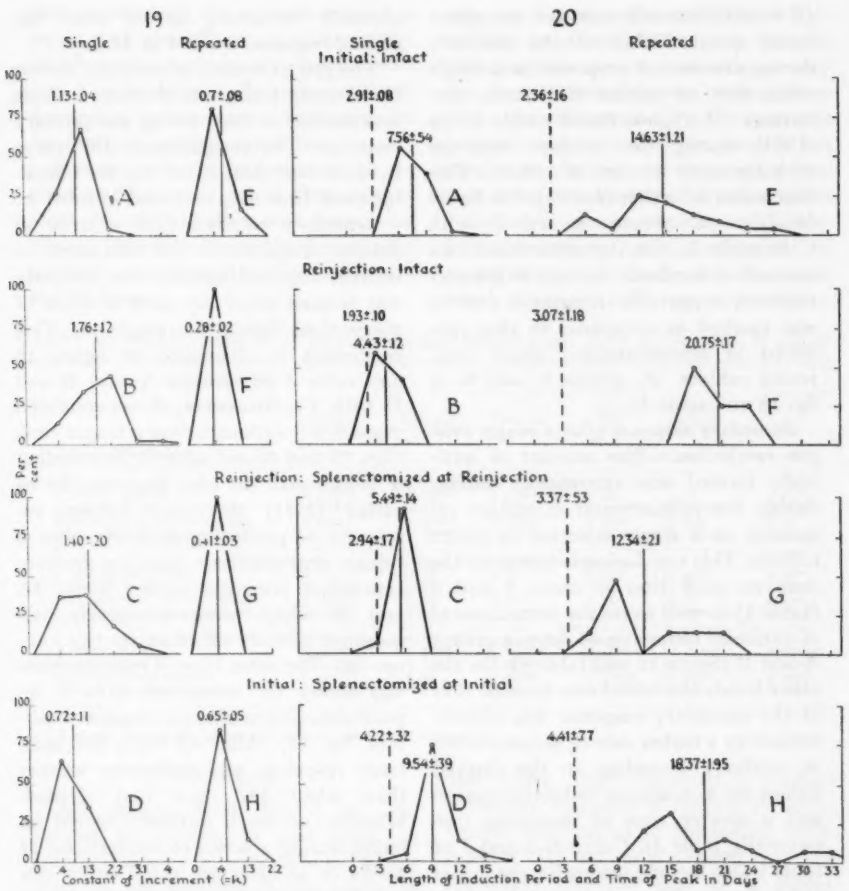


FIG. 19.—Percentage frequency distributions for the rate of accumulation (k) to peak titer with means and standard errors for the same groups of rabbits as in figure 18. A derived single value for k was used for the reinjected groups and for all initial rises having two or more phases.

Note that the values for k to peak titer are small for the splenectomized rabbits after a single initial reinjection (group D) and for all rabbits repeatedly injected and reinjected (groups E to H).

FIG. 20.—Percentage frequency distributions for the total time from injection or reinjection to peak titer (solid lines) with means and standard errors for the same groups of rabbits as in figure 18. Means (dash lines) and standard errors for the length of the induction period are also included.

Note that the induction period is shorter after reinjection in intact singly injected rabbits but not in repeatedly injected intact ones. The same holds true for time to peak titer.

able intact rabbits in series 1 and splenectomized rabbits in series 2 were analyzed by Student's method of paired comparisons, the intact animals attained a mean of 1750 ± 582 more hemolytic units/ml than did the splenectomized animals. This difference is not only highly significant statistically

($P = 0.001$) but indicates that the spleen forms about 71.1% of the antibody during the second response to a single reinjection or about the same percentage (75.8%) as found earlier by us (1951) during the primary response with the same amount of antigen. This conclusion is further illustrated in figure 18 (also cf. groups A and B with C in table 3). On the other hand, the amount of antibody formed in splenectomized, repeatedly reinjected rabbits was marked as compared to that produced in splenectomized singly reinjected rabbits (cf. groups C and G in fig. 18 and table 3).

Secondary response after a single antigen reinjection.—The amount of antibody formed was statistically similar during the primary and secondary responses to a *single* injection in intact rabbits. This conclusion is based on the data on peak titer in series 1 and 3 (table 1) as well as on the total amount of antibody formed in 30 days in groups A and B (figure 18 and table 3). On the other hand, the initial rise to peak titer of the secondary response was characterized by a higher rate of accumulation of antibody according to the derived values for k, a shorter induction period and a shorter time of formation (see especially table 3). Columns 5 and 7 of table 1 also include means for the initial antibody rise. These data may be of interest should later work prove that the spleen is only involved in this phase. They give about the same statistical validity as corresponding means for peak titer and the rate of its accumulation.

Intense antigenic stimulation during initial immunization did not cause any significant enhancement of the response to a later single antigen injection since the secondary response was never more intense than would be expected had the rabbits been injected only once initially. On the contrary, it probably produced a partially refractory period since the second response was low at 42 days.

The rate of decline of antibody during the secondary response in intact animals was similar to that during the primary response. The insignificant differences between the most rapid decline following peak titer may be found in table 2.

Secondary response after a series of antigen reinjections.—As compared to initially injected rabbits, less antibody was formed relatively after a series of reinjections than after a single one. This conclusion is illustrated in figure 18 and table 3 (cf. groups A with B and E with F). Moreover, it accumulated more slowly although over a longer time (figs. 19 and 20 and table 3). This finding is in line with the idea expressed by us earlier (1951) that sites become refractory to producing antibody after a certain degree of immunization until regenerative processes occur. Series 5A and B which were intensively immunized initially substantiate this contention. The same type of refractoriness was shown for nonsplenic sites in repeatedly reinjected splenectomized rabbits (fig. 11). After 42 days, the antibody response was uniformly weaker than when 167 days had elapsed. Whether as much antibody would be found during a series of reinjections 24 weeks or so after an initial series can only be answered after further work.

The rabbits of group G were treated identically like those of group F except that they were splenectomized at the time of the first of the second series of immunizing injections. Although titers were too variable and the groups of rabbits too small to give statistically significant differences (table 3), more antibody was probably formed (fig. 18) at a slightly faster rate (fig. 19) and in a shorter period of time (fig. 20) in group G than in group F. It would seem that two antagonistic factors may be operating here, i.e., the partially refrac-

tory state which the animals seemed to be in and the effects of splenectomy, which we have previously shown (1951) has a stimulating effect on antibody formation during intense immunization by multiple antigen injections.

Serum antibody had started to decline at 30 days in all of these rabbits even though injections were being continued. One-fifth to one-third more total antibody was formed during the following 30 days.

response is not as characteristic of particulate as of soluble antigens. Results with particulate microbial antigens vary but Burnet and Fenner (1949) believe that the differentiation between the primary and secondary response is not nearly as definite as for toxins and toxoids. They reported practically no secondary response to antigens consisting of relatively pure suspensions of rickettsias of Q fever. On the other hand, Topley (1930, see also Wilson

TABLE 3.—*Differences with the statistical significance† between means shown in figures 18, 19 and 20.*

From figure		20		18		19
Difference between:‡	Groups	Induction period	Time to peak	Peak titer	Total antibody for 30 days	k
				$\times 10^3$ units/ml		
Response after single injection or reinjection						
I and R: both intact	A and B	0.98***	3.13***	0.63	1.01	0.63***
I and R: R splenectomized	A and C	0.03	2.07***	2.19***	2.84***	0.27**
R in intact vs. splenectomized	B and C	1.01***	1.06***	1.56**	1.83*	0.36
I in intact vs. splenectomized	A and D	1.31***	1.98***	2.14***	2.74***	0.41***
Response after a repeated series of injections or reinjections						
I and R: both intact	E and F	0.71	6.12*	10.87*	25.42**	0.42*
I and R: R splenectomized	E and G	1.01*	2.29	5.29	15.0	0.29
R in intact vs. splenectomized	F and G	0.30	8.41*	5.58	10.42	0.13*
I in intact vs. splenectomized	E and H	2.05	3.74***	19.26**	38.41*	0.05
Response after single injection compared with response after repeated injection						
I: both intact	A and E	0.55**	7.07***	12.48***	34.94***	0.43***
R: both intact	B and F	1.14*	16.32***	2.22**	10.33***	1.48***
R: both splenectomized	C and G	0.43	6.85***	9.38***	22.78**	0.99**
I: both splenectomized	D and H	0.19	8.83***	33.88***	76.09***	0.07

† *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$.
‡ I, initial response; R, anamnestic response after a test reinjection.

DISCUSSION

The present work should be considered in relation to past work on specific anamnestic responses and to our work on the function of the spleen in antibody production.

It is generally agreed that antibody response is less active on first contact with an antigen than after second or subsequent contacts. The classic example of a pronounced secondary response involves diphtheria toxin (see review by Glenny and Südmerson, 1921, and Glenny, 1931) and is characterized by a shorter induction period and a steeper rise to a higher titer as compared to the primary response. Various authors have stressed the fact that a pronounced secondary or homologous anamnestic

and Miles, 1946) reported a marked secondary response in rabbits to intravenous suspensions of *Salmonella paratyphi* B. He not only stressed that the secondary response rose to a higher titer, but that the high titer was maintained for a longer period than after the peak of the primary response.

Burnet and his coworkers (see Burnet and Fenner, 1949) have differentiated the character of the primary and secondary rise of antibody formed in response to a soluble antigen. According to them, a plot of log titer against time gave a sigmoid curve during the slow rise of the primary response and a straight, steep line during the secondary rise. Burnet's and our results need not be incompatible. We frequently found two

or three linear phases for the initial antibody response, each with a successively decreasing value for k, and usually a single phase for the second response. Some of Burnet's sigmoid curves could have been drawn as discontinuous straight lines just as many of our multiphase rises could have been drawn as sigmoid curves except that closely spaced antibody titrations convinced us that they consisted of discontinuous "compound interest" curves which broke sharply (our work, 1950). We did not obtain a longer persistence of antibody in intact animals as found by Topley with paratyphoid vaccines.

The overwhelming evidence in the present work indicates that the spleen plays about the same role in producing antibody in response to a second antigen injection as to the first one. Not only is the spleen of paramount importance in forming most of the antibody present at peak titer, but it abruptly ceases to form antibody at about peak titer while nonsplenic sources continue to form small amounts. The most direct evidence for the cessation of splenic activity is the finding that splenectomy at about the time of peak titer did not increase the rate of antibody disappearance as it would had the spleen been pouring appreciable amounts of antibody into the serum. We did not splenectomize animals at about peak titer of the secondary response, but the general rate of antibody decline after the secondary peak titer in both splenectomized and intact animals indicates that the behavior of the spleen is the same in both responses.

Our previous work (1950 and especially 1951) showed that nonsplenic sites become relatively more active than the spleen as antigenic stimulation is increased. When multiple injections of antigen were given, nonsplenic sites were more active in splenectomized rabbits than nonsplenic sites plus the spleen in intact rabbits. The results obtained in experiment 4 of the present work leave little doubt that the spleen is relatively unimportant during the secondary response induced by multiple injections. Thus, intact rabbits in series 6 secondarily immunized with multiple injections produced about the same amount of antibody as rabbits in series 7 similarly treated but splenectomized at the beginning of the second series of antigen injections.

In series 3 and 6, nonsplenectomized operative controls (appendix, one kidney or one testis removed) sometimes gave higher anamnestic responses than intact animals. This result was also reported by us (1951) for the initial response. We have no satisfactory explanation for the apparent persistence of this effect in rabbits 155, 157 and 159 in series 6 which were reinjected 70 to 89 days after the operation.

Recently we (1951) published evidence for believing that the spleen and possibly all antibody-forming sources form a certain amount of antibody and pass into an inactive phase. As a working hypothesis, we also suggested that the speed at which a site like the spleen enters and recovers from the inactive phase depends upon such interrelated factors as (1) rate of antibody formation, (2) availability of anabolic intermediates and (3) presence of antigenic stimulation. We have, for convenience, divided this phase into (1) the inactive phase proper which is limited to situations in which the site produces more antibody when more antigen is injected and (2) the refractory phase in which it does not. The limiting factor of the inactive phase is apparently the presence of antigen and that of the refractory phase is most likely the postulated anabolic intermediates. Such intermediates are probably concerned

in that phase of normal globulin synthesis which can be redirected to produce specific antibody.

The present results are in agreement with the foregoing hypothesis. If the cessation of antibody formation, i.e., the inactive phase, after a single antigen injection in the amounts we used, is due to lack of antigenic stimulation, it is not unexpected that a single reinjection from seven days on should result in the formation of about the same amount of antibody and in about the same proportion by the spleen and nonsplenic sites. This is what we found. On the other hand, rabbits given a single reinjection after a series of initial injections were in a partially refractory phase 42 days after the initial immunization whereas the reactivity was regained by 167 days (series 5). It is interesting that the anamnestic response thereafter only attained and did not exceed that following a single initial injection.

Previous immunization after a single antigen injection shortens the induction period of the secondary response. On the other hand, splenectomy (compare the initial response of series 8A and 8B with that of series 3 and 4 in table 1) or large doses of X rays lengthen it (Taliaferro et al, 1952). When splenectomy is combined with a second injection of antigen, the two factors largely cancel out. The shortened induction period and possibly the increased rate of antibody accumulation are probably associated with a greater concentration of anabolic intermediates. These intermediates may be present from the primary response or may be secondarily associated with the more rapid absorption of antigen from the blood and deposition in the antibody-forming sites (cf. Talmadge et al, 1951). In this connection, it is noteworthy that the induction period of the secondary response was not shortened (3.4 days) in the rabbits of series 5A, which we believe were in a partially refractory stage from the initial intense immunization, whereas it was shortened (1.8 days) in the rabbits of series 5B after normal reactivity was regained.

The anamnestic response in rabbits splenectomized at various intervals during initial immunization are chiefly of value in indicating that partial and possibly, at times, full physiological compensation for the lost spleen occurs in the rabbit. Thus, in figure 14, some of the rabbits which had been splenectomized 90 to 126 days prior to the second injection of antigen gave responses that would be expected in intact animals. The same is true of the rabbits in series 9B (fig. 15), which were splenectomized 193 days before the test series of reinjections. These results are in line with our previous finding (1950) that two of three rabbits splenectomized 28 days before and three rabbits splenectomized 98 before a single initial injection of red cells gave essentially normal responses.

CONCLUSIONS

The anamnestic response was studied in 39 intact and 55 splenectomized rabbits, as well as in 16 rabbits from which the appendix, one testis or one kidney was removed at the time of the first injection. Single or a series of repeated reinjections of standardized suspensions (varying over an 80-fold range) of sheep red cells was used. As in our previous work, all titers were determined photometrically in 50% hemolytic units.

1. A single reinjection of sheep red cells at intervals from 7 to 176 days after a similar initial injection stimulated the formation of statistically similar amounts of antibody as the initial injection, but hemolysin began to be formed

sooner (induction period shortened by a third) and at a more rapid rate (the derived constant of increment about doubled). In addition, the rate of the anamnestic rise to peak titer usually consisted of one rapid rise, whereas the initial rate of rise to peak titer frequently consisted of two phases. The spleen formed about 71% of the antibody at peak titer after reinjection, which is approximately equal to the amount formed during the initial response. No evidence was found that hemolysin declined after the second response at a slower rate than after the peak of the primary response.

2. Intense initial immunization by multiple antigen injections induced a partially refractory phase to a single reinjection at 42 days. Recovery thereafter (between 42 and 167 days) was associated with the production of hemolysin within but not above the normal range for the initial response after a single antigen injection.

3. A second multiple series of injections of red cells at intervals varying from 21 to 64 days after the last of a similar series of initial injections in four rabbits incited a greater secondary response than a single reinjection of antigen but not as great as the initial response during the series of initial injections. These animals were probably in a partially refractory phase from the initial multiple immunization. Splenectomy at the beginning of the second series of antigen reinjections did not depress antibody formation as it did at the time of a single reinjection. This lack of effect of splenectomy agrees with our previous finding that the spleen becomes relatively less important as antigenic stimulation is increased.

4. More hemolysin was sometimes produced both primarily and secondarily in rabbits from which the appendix, a testis or a kidney had been removed on the day of the initial injection of red cells than in intact singly injected rabbits and intact or splenectomized repeatedly injected ones.

5. Nonsplenic sites partially compensated for the loss of the spleen in rabbits splenectomized 51 days or more prior to a single reinjection of red cells.

6. The characteristics of the anamnestic response are compatible with the suggestion made previously for the primary response, viz., that the spleen and possibly all antibody-producing sites form a certain amount of antibody and then pass into an inactive and, at times, a refractory phase during which they form little or no antibody.

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MAEDI, A CHRONIC, PROGRESSIVE INFECTION OF SHEEP'S LUNGS

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During the last twelve years or so a lung disease of unknown etiology has been prevalent in sheep in Iceland. Except for a general emaciation, distinctive anatomical changes in this disease are found only in the lungs, which show a chronic pneumonia, and in the tracheobronchial lymph nodes. Although the cause of this disease is not known, all circumstantial evidence suggests that it is contagious, and that it has a very long incubation period—one to three years or more. It was probably introduced into this country in 1933 with some rams of karakul breed which were imported from Germany at that time. Two other diseases which were undoubtedly introduced with these rams have been described earlier (Dungal et al, 1938; Sigurdsson, 1945), so that this seems to be the third disease introduced with this flock.

The disease in question is in Iceland called maedi. It is very prevalent in many districts in Iceland and causes extremely heavy losses to the farmers, as it is not unusual that 20 to 30% of the flock may succumb annually. It is rarely observed in sheep less than two years old, but from that age on losses are often heavy.

In the absence of any other methods of control this disease has in recent years been dealt with under a slaughtering policy in certain districts. All the sheep in a given district have been destroyed, and the lambs from an uninfected area have been introduced in their place. So far about 170,000 sheep have been destroyed according to this plan, and about 80,000 healthy sheep have been introduced into the same areas again. There remain in infected areas about 130,000 sheep which will have to be destroyed if the slaughtering policy is to be continued.

It is still too early to conclude that these measures have succeeded in eradicating the disease from the areas in question, and any attempts to predict this would be futile, as the mode of spread of maedi is not known. If the disease does not reappear in the next few years in the areas where an exchange of the stock has been made, it is likely that the sheep in the remaining infected areas will also be destroyed.

The present paper will not discuss the epidemiology of maedi, as this aspect has been more fully investigated by others, but we shall instead confine ourselves to some clinical and laboratory studies on this new disease.

CLINICAL STUDIES

The first signs of maedi are usually a slowly advancing listlessness and loss of condition. This often becomes apparent when the weather changes for the worse, or after the sheep have been exposed to some unusual physical strain such as long driving. An early sign is that the sheep become dyspnoeic, and after an exertion the respiration becomes very rapid and shallow. It is not possible to distinguish in the individual case be-

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tween maedi and any other kind of afebrile pneumonia, but if the history of the case is known, the protracted course and progressive loss of condition in a flock where maedi has occurred are suggestive. In contradistinction to epizootic adenomatosis (Dungal et al, 1938) maedi does not produce in the lung any appreciable quantity of fluid which could be poured out through the nostrils.

The frequency of respiration is usually above normal and sometimes excessively high (80 to 120 per minute at rest). As the disease progresses the respiration becomes very difficult or labored, the nostrils are dilated and flank breathing or pumping is commonly observed, even while the animal is at rest. In some cases coughing with more or less nasal discharge has been observed.

The body temperature in uncomplicated cases is within the normal range, although possibly close to the upper limit. This has been observed repeatedly over a number of years. The same may be said of the pulse rate, although this is rather difficult to determine in sheep, as the handling involved tends to influence the rate of the pulse.

As the disease advances and the sheep lose condition the hemoglobin content of the blood decreases. This is not an early sign, but values as low as 7 to 8 g hemoglobin per 100 ml blood are sometimes found in late stages. The normal value for hemoglobin in sheep in Iceland is from 12 to 14 g per 100 ml blood.

The number of red cells also decreases but proportionately less than the hemoglobin content, so that the anemia is of the hypochromic type. The mean corpuscular hemoglobin value is normally from 11 to 13, but in cases of anemia due to maedi it is usually between 10 and 11.

The number of white cells in the blood of maedi cases varies generally from 6 to 12 thousand per mm^3, averaging about 8.5 thousand per $mm.^3$ This is slightly above normal as the normal figures found here are usually from 4 to 6 thousand per mm^3.

Nonprotein nitrogen of blood plasma was found to be normal. The average and standard error for eight diseased sheep was 31.5 ± 1.7 mg per 100 ml plasma, and for a control group of seven clinically healthy sheep 31.4 ± 1.3. These determinations were carried out according to Koch and McMeekin (1924) after deproteinization of the plasma with tungstic acid.

Total protein was determined in plasma from 19 diseased sheep. The average for these determinations was 6.05 ± 0.12 g protein per 100 ml of plasma. A control group of 20 clinically healthy animals gave the average 6.50 ± 0.08 g protein per 100 ml of plasma. These determinations were carried out by a micro-Kjeldahl method with direct nesslerization (Koch and McMeekin, 1924; Miller and Miller, 1948). A correction for nonprotein nitrogen was introduced. The nitrogen content of the protein was assumed to be 16%. The lowest of the values determined, 4.7 and 4.8 g protein per 100 ml plasma, were found in the plasma from two of the diseased animals. Both of these sheep, and only these two, showed hepatitis upon autopsy. The highest value in the diseased group was 7.2. The probability, P, that the difference in the two averages is caused by chance alone, was calculated to be far below 0.01, whether the two sheep suffering from hepatitis are included or not. The average total protein in plasma in the diseased group thus turns out to be significantly lower than the average for the control group.

Preliminary analysis of the albumin-content of plasma after fractionation with sodium sulfate indicates that the disease is accompanied by hypoalbumi-

nemia, which would account for the low total protein of the plasma while the globulin is near its normal value. The low values for total protein are possibly an indication of the general wasting of the organism encountered in advanced cases of maedi. This may be compared with the emaciation found in malignant disease and chronic infections.

Fibrinogen was determined in plasma from 19 diseased sheep. The average found was 0.359 ± 0.022 g fibrinogen per 100 ml of plasma. A control group of 10 sheep of approximately the same age distribution as the former, gave an average of 0.297 ± 0.014 g fibrinogen per 100 ml of plasma. For the difference in these two averages P was calculated to lie between 0.10 and 0.05. The difference thus cannot be considered as significant. It was found essential to take into account the age factor, since 10 healthy yearlings were found to have significantly lower fibrinogen than the control group, i.e., 0.216 ± 0.013 g fibrinogen per 100 ml plasma. The determinations were carried out by the same micro-Kjeldahl method as was used for the determination of total protein. Fibrinogen was isolated as fibrin (Cullen and Van Slyke, 1920) and brought into solution with a minimum amount of 1% solution of sodium hydroxide.

Reducing sugars in blood plasma were found to be normal. The average for 6 diseased sheep was found to be 62.3 ± 1.9 mg per 100 ml of plasma, calculated as glucose. The average for five control sheep was 63.8 ± 1.1 mg. per 100 ml of plasma. The determinations were done on protein-free plasma filtrate according to Benedict (1931). Venous blood was drawn 14 hours after the last meal and the plasma was deproteinized with tungstic acid.

The carbon dioxide capacity of blood plasma was determined in plasma from 16 diseased sheep. The average for these determinations was 70.0 ± 1.2 volumes percent. A control group of 15 sheep gave the average 64.2 ± 0.9 volumes percent. These determinations were done manometrically in the Van Slyke and Neill manometric gas analysis apparatus, after saturation of the plasma with alveolar air. A calculation of the significance of this difference gives P much lower than 0.01, and this increase in carbon dioxide capacity is therefore considered significant.

The increased carbon dioxide capacity of the plasma suggests in this case acidosis of respiratory origin. The lung damages may inhibit the normal release of carbon dioxide from the blood and give an increased carbon dioxide tension in the plasma. This would stimulate the compensatory mechanisms and lead to an increased carbon dioxide capacity.

It is doubtful that recoveries ever occur in maedi, and they are certainly rare. General practice among the farmers is to destroy all cases encountered in the fall and until mid-winter. Those that occur after March or so they let live in the hope that they will be able to rear a lamb, which they sometimes do. Under natural conditions cases of maedi very often succumb to a terminal acute pneumonia, usually showing large numbers of the bacilli described by Dungal (1931). If the sheep are not killed and if they meet with no particular hardships they may usually be expected to survive for three to six months after the first symptoms are noticed. In certain cases symptoms last much longer, even for years.

FINDINGS AT AUTOPSY

Macroscopic findings.— Uncomplicated cases of maedi show no typical pathological macroscopic changes outside the thoracic cavity. The carcass is usually thin, and there may be some

serous fluid in the abdomen. When the thorax is opened the lungs collapse less than normally, and in a minority of cases fibrous adhesions are found covering a portion of the surface of one, or occasionally both, lungs.

The most striking fact about the diseased lungs is their size. Instead of the normal weight of from 300 to 500 g the maedi lungs weigh from two to five times that much. The tracheobronchial lymph nodes are also greatly enlarged. Table 1 gives the weight of the lungs and of the tracheobronchial lymph nodes in 10 consecutive cases of maedi autopsied in our laboratory.

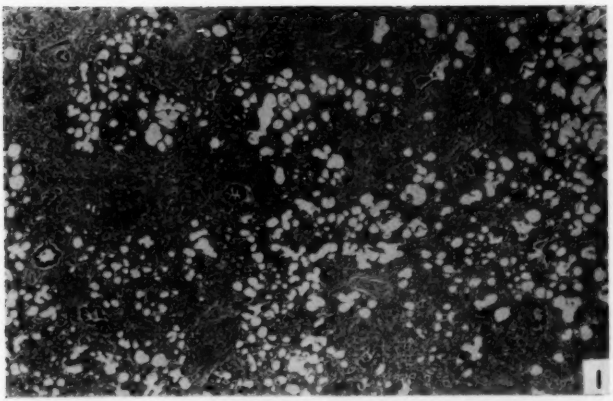
TABLE 1.—*Weight of lungs and tracheobronchial lymph nodes in 10 cases of maedi in sheep (normal weight of lungs about 300 to 500 g; normal weight of lymph nodes about 10 to 15 g).*

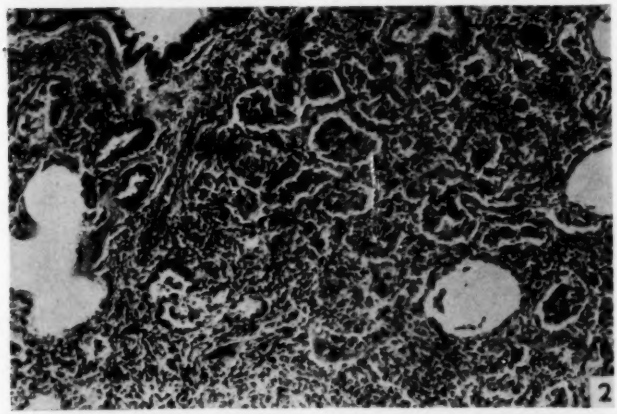
Sheep no.	Weight of lungs in grams	Weight of tracheobronchial lymph nodes in grams
173	1120	30
174	1140	28
175	1330	45
176	1160	56
177	860	
179	1040	24
181	1810	34
182	970	65
183	1800	48
184	950	26
Average	1218	39.5

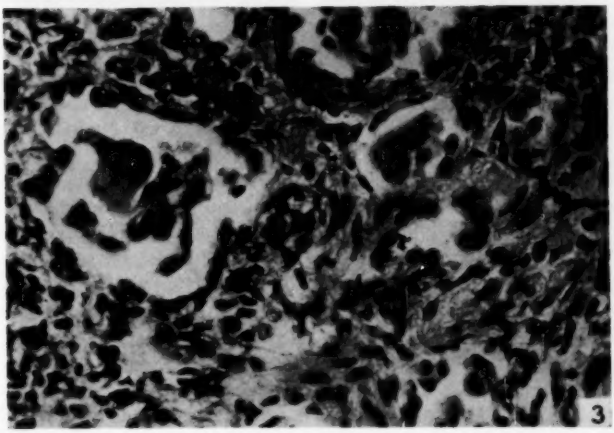
The color of the maedi lungs differs considerably from normal lung color. It varies from greyish yellow to greyish blue. To the touch the diseased lungs are diffusely thickened and feel somewhat like a rubber sponge, although less elastic. The whole lungs are usually remarkably uniform in color and consistency, i.e., the pathological changes seem to progress evenly in the whole organ with no normal lung tissue visible anywhere. This is in contrast to other lung lesions with which we are familiar, where diseased and normal parts may be easily distinguished in the same pair of lungs. On the cut surface the tissue is rather dry, and it is homogenous as to color and consistency there also. The larger bronchi often contain some mucus. It should be emphasized that the consistency of the lung tissue in maedi is always very much softer than in ordinary pneumonic hepatization.

Microscopic findings.—The main characteristic of the histological picture in maedi is a proliferation of mesenchymal tissue with a thickening of the alveolar septa, and at times numerous macrophages in the alveoli (figs. 1, 2, and 3). Large solid areas are usually not seen in the slides, but in advanced cases the air spaces sometimes appear as round or oval openings in the lung tissue, which has to a great extent lost its characteristic structure and is transformed into strands of mesenchymal cellular tissue. In such cases much more area in a given field is covered by the solid tissue than by the air spaces. Presumably the alveolar septa have given rise to the bulk of the pathological tissue that is seen in the microscope. This would be concluded from the appearance of the tissue in young cases rather than from the structure of the lung in advanced cases, where it is no longer possible to recognize many of the mesenchymal strands as septa. In young cases the alveolar septa seem hyperemic, but later in the disease there is a considerable increase in fibrous tissue and the smooth muscle normally found at the level of the alveolar ducts appears in many cases hypertrophic.

In some cases of maedi microscopically visible accumulations of lymphoid tissue are found scattered through the lung. These accumulations are at times very conspicuous and are particularly to be found close to a bronchus or a bronchiolus. It is, however, difficult to assess the significance of this finding, as in our experience accumulations of lymphoid tissue are frequently found in normal sheep's lungs, although we







FIGS. 1–3.—A typical maedi lung. For a description of the lesion see the text. Fig. 1, ×14. Fig. 2, ×130. Fig. 3, ×480.

believe it is particularly conspicuous in maedi.

The cellular exudate in the alveoli is composed mainly of the common alveolar macrophage. A few lymphocytes are also seen, but few if any segmented leucocytes appear, except in cases where an acute pneumonia has supervened, when they are, of course, extremely numerous.

In Giemsa-stained smears from maedi lungs we find almost always large mononuclear cells with protoplasmic inclusions of a rather typical appearance (fig. 4). They are almost perfectly round, varying in size from 1 to 3 μ, and they stain with a soft greyish blue color that is rather characteristic. They are seen either single or multiple within a cell. If multiple they may vary considerably in size. We have not seen these inclusions in normal lungs, but we have not searched for them in a sufficient number of other pathologically changed lungs to be able to maintain that these inclusions are pathognomonic for maedi, although with the evidence at hand this would seem to be so.

The bronchial tree is in general remarkably free of lesions, the epithelium usually being intact and the lumina free of exudate. It is a common finding, however, to see small epithelial formations outside the bronchi in the form of irregular cavities lined with cubical or cylindrical epithelial cells. These epithelial formations are never very extensive and remind one of the findings in influenza as reviewed and extended by Loosli (1949).

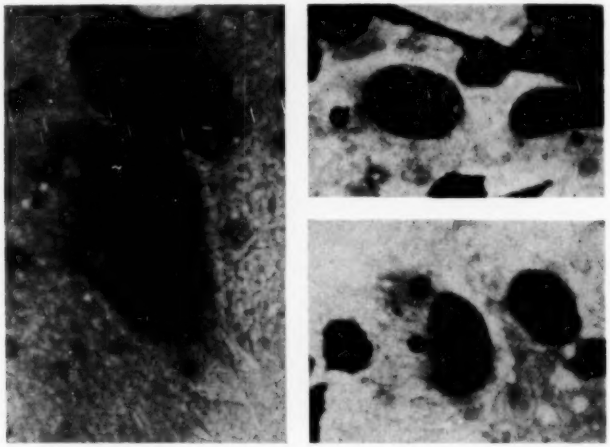


FIG. 4.—Giemsa-stained cells in smear preparations from a maedi lung. ×1575.

The enlarged tracheobronchial and mediastinal lymph nodes show a generalized hyperplasia and chronic inflammatory changes.

Inflammatory changes in the livers have been found in several advanced cases of maedi. These appear as small, scattered infiltrates of lymphocytes and plasma cells without apparent connection with the vessels of the liver.

Supplementing our historical observations we determined the amount of connective tissue in four maedi lungs and four normal lungs by digesting in 0.1 *N* sodium hydroxide (Warren and Wahi, 1947) and drying and weighing

the insoluble, fibrous residue. This method is admittedly rather rough, but the results shown in table 2 indicate that on a dry matter basis the amount of fibrous tissue is approximately the same in infected and in healthy lungs, the average of fibrous tissue being 14% and 12%, respectively. The table also shows that there is about the same amount of dry matter per unit weight of wet substance in the diseased lungs as in the normal lungs.

TABLE 2.—*Amount of fibrous tissue and percentage of dry matter in four maedi lungs and four control lungs.*

Lungs no.	Before digestion			Put in digestion (mg)	Fibrous tissue remaining after digestion		
	Wet weight in grams	Dry weight			Mg	Percent of dry matter	Percent of wet matter
		Grams	Percent of dry weight				
			Maedi-infected sheep				
73	5.0	1.135	22.7	500	68.0	13.6	3.1
74	5.0	1.139	22.8	500	42.6	8.5	1.9
76	5.0	1.145	22.9	500	63.1	12.6	2.9
77	5.0	1.370	27.4	500	65.5	13.1	3.6
Average		1.197	23.94		59.8	12.0	2.87
			Control sheep				
173	5.0	1.040	20.8	500	62.8	12.6	2.6
176	5.0	1.000	20.0	500	66.4	13.3	2.7
181	5.0	1.032	20.6	500	52.8	10.6	2.2
184	5.0	1.087	21.7	500	97.9	19.6	4.2
Average		1.040	20.77		70.0	14.0	2.91

ETIOLOGICAL STUDIES

The experience in the field seems to indicate that maedi is transmitted from sick to healthy sheep by contact. Our experimental results which will not be reported here also indicate this.

We have not, however, been able to demonstrate any etiological agent responsible for this infection. The lungs from uncomplicated cases of maedi are commonly found to be sterile when inoculated on the ordinary plain or enriched bacteriological mediums (blood-agar, made with horse blood, chocolate agar, tryptose agar) and incubated aerobically or anaerobically. Ten consecutive cases of maedi which were recently autopsied were studied by grinding pieces of lung tissue in a glass grinder with sterile distilled water to make a 20% suspension. This suspension was then inoculated on plates of plain agar, blood agar and chocolate agar, two drops on each plate. Five days later an average of three colonies was found on each plate, but these colonies were the common contaminants.

Attempts to cultivate fungi on Saboraud's medium have also given negative results.

Dr. C. E. Roach of the Lilly Research Laboratories, Indianapolis, Indiana, kindly furnished samples of histoplasmin, blastomycin, toxoplasmin and torula antigen for intradermal tests. Dr. Charles E. Smith of Stanford University School of Medicine, San Francisco, California similarily furnished samples of coccidioidin. Each antigen was tested intradermally on from 11 to 25 proven or suspected cases of maedi with negative results. This would seem to exclude fungi and the tortula yeast as factors of etiological significance in maedi.

Serums from six cases of maedi were subjected to complement fixation tests with antigens specific for psittacosis and Q fever. Five normal serums were tested in the same way at the same time as controls. The tests for psittacosis were negative. Some of the serums showed a slight fixation with the Q fever antigen, but this was true of both the maedi serums and the controls.

Lungs from cases of maedi are generally no more heavily infected with lung worms than are lungs of other sheep in Iceland, nor are there other known reasons to suspect that metazoa may play a role in the etiology of this disease.

If an insect vector for the hypothetical infectious agent were to be considered, it may be pointed out that the sheep ked (*Melophagus ovinus*) is almost universally present on Icelandic sheep.

As no bacteria or fungi have been found that could be suspected of causing maedi, we have attempted to demonstrate a virus in the infected lungs. These attempts will be reported later.

We have also attempted to demonstrate a specific antigen in lungs of infected sheep. For this purpose we have prepared in various ways numerous extracts from infected lungs and used them in the complement fixation test with serums from natural cases of maedi. We have not succeeded in demonstrating conclusively the presence of a specific antigen in maedi tissue.

We have tested the effect of aureomycin* on five cases of maedi. The drug was administered intravenously as follows: The initial dose was 5 mg per lb of body weight. After that 2.5 mg per lb of body weight were administered every 12 hours for four days. A second course of exactly the same treatment was given again ten days later. Five similar cases of maedi were kept as controls. The treated animals showed no clinical signs of recovery. The frequency of respiration continued to increase in both groups, the hemoglobin values of the blood fell at about the same rate in both groups, etc.

The sheep were slaughtered and autopsied about two months after the treatment was begun. They were all found to be typical cases of maedi, and no striking differences between the weight or general appearance of the lungs in the two groups were observed.

Although the infectious nature of maedi seems beyond question, we have considered the possibility that a nutritional deficiency might be involved. We have no evidence that such is the case. In particular we have considered the possibility that a deficiency of the microelements, copper and cobalt, might be involved. Several serums of sheep on an affected farm were analyzed for copper. The values found varied from 0.09 to 0.12 mg copper per 100 cc of blood, which is within the normal range.

Samples of soil and herbage from two affected districts were analyzed for cobalt. The average values from the first district were 2.07 parts per million cobalt in the soil and 0.24 p.p.m. in the herbage, and from the second district 7.8 p.p.m. in the soil and 0.29 p.p.m. in the herbage. A nutritional deficiency of cobalt would not be expected to occur in animals grazing on land showing these values for cobalt.

Extensive feeding experiments with copper and cobalt did not indicate that these microelements had a beneficial effect on clinical cases of maedi, nor did they prevent the occurrence of new cases in flocks where these minerals were being fed.

DISCUSSION AND SUMMARY

A chronic pneumonia of sheep is prevalent in many districts of Iceland. The epidemiological behavior of the disease, which in Icelandic is called maedi, definitely indicates that it is an infectious process. The cause of the disease is

* Obtained through the courtesy of Lederle Laboratories Division, American Cyanamide Company, Pearl River, N. Y.

not known, but it is believed that microscopically visible organisms have been ruled out as a possible cause. The disease is characterized by an incubation period of about two years and a protracted course. The lungs of a typical case weigh from 800 to 2000 g instead of the normal weight of 300 to 500 g. Anatomically the disease consists principally of a diffuse mesenchymal proliferation in the lung. The bronchial and mediastinal lymph nodes are also enlarged.

Other clinical and laboratory characteristics of this disease are discussed in the present paper. No indication was obtained in these studies that the disease is primarily a metabolic or nutritional disorder.

The etiology of maedi is not known, although a virus infection is suspected. No serological tests are available to distinguish it from or identify it with other diseases of sheep lungs.

In the absence of such definite etiological information it seems futile to discuss at the present time the possible relationship of maedi to other lung diseases of sheep. We want to point out, however, that typical cases of infectious adenomatosis or jaagziekte, which is a well known disease of sheep lungs occurring in this country and elsewhere, are distinctly different from maedi both anatomically and as regards the clinical picture.

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IMMUNIZATION AGAINST SALMONELLA INFECTION IN A BREEDING COLONY OF MICE

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An attempt was made to determine the effect of immunization in two colonies of breeding mice against an epizootic of *Salmonella enteriditis*. The mice were of CFW strain, which is extremely susceptible to salmonella infections. Mortality is nearly 100% and the carrier incidence is low. All mice were bred in room 2, described later, removed to a distant room at weaning time (17 to 24 days of age), and stored there until returned to the experimental rooms as breeders at 8 to 10 weeks of age.

MATERIALS AND METHODS

The organism, which had caused an epizootic in one colony, was identified by the New York Salmonella Center as *Salmonella enteriditis* and given the culture number 4185. The MLD by intraperitoneal injection was determined to be 500 or less organisms.

The bacterin used was Typhoid Combined Vaccine (TCL),* containing *Eberthella typhosa*, *Salmonella paratyphi*, and *Salmonella schottmuelleri*. This preparation was suggested as it contained the somatic antigens for group B and D salmonellae. It was also suggested that minimal doses be used in the attempted immunization. Accordingly, the individual dose was set at 1/10 ml of a 1:1000 dilution of the original vaccine, giving a dose of 100,000 *typhosa*, 50,000 *paratyphi* and 50,000 *schottmuelleri*. Two intraperitoneal doses were given one week apart, and one week later the mice were removed from storage to the experimental room, at 8 to 9 weeks of age. Experiments with limited numbers of animals showed that the bacterin at this level did not protect against the organism when introduced intraperitoneally.

The experiment was conducted in two rooms, numbered 2 and 3, which were physically identical in every respect; each had one outside wall with the same exposure; air supply and heat were delivered through a common duct which divided outside the rooms and entered each room at the same point; a single thermostat controlled the air temperature. The interior fittings were identical, consisting of four racks, each holding 120 boxes. Each box held a family group of five females and one male, giving a total of 720 mice to a rack. The control groups, each 720 animals, were placed on the rack along the north wall of each room. The other three racks were filled with the treated mice.

The final arrangement of animals in each room was as follows:

Room 2

- 1 rack: 720 untreated animals (group A, control); replacements of dead animals were marked for identification and *treated* (group B, experimental)
- 3 racks: 2160 treated animals (group C, experimental); replacements of dead animals marked and *treated* (group D, experimental)

Room 3

- 1 rack: 720 untreated animals (group E, control); replacements of dead animals marked and *untreated* (group F, control)
- 3 racks: 2160 treated animals (group G, experimental); replacements of dead animals marked and *treated* (group H, experimental)

There was, however, a difference between the two rooms, the importance of which was not realized at the time and that was the way in which they were established. Room 2 was the room in which the disease was in severe epizootic form at the time the experiment was started. To preserve the epizootic until the experimental arrangement was completed, this room was reduced to a single rack of untreated mice which later became control group A. As large blocks of the remaining untreated mice were removed, the space which they had occupied was thoroughly cleaned, the shelves washed with a chlorine solution (1000 parts per M), and after

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The authors are deeply indebted to Dr. A. J. Weil for advice prior to the experimentation, to Dr. Joseph F. Kubis for assistance in the experimental design and for statistical evaluation, to Dr. Colin M. MacLeod and Miss Norma Styron for lethal dose determinations, and to Dr. H. C. Batson for statistical computations.

* Commercial bacterin supplied through the courtesy of American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y.

one week the space was filled with treated mice in clean boxes, group C. The older mice in the untreated control block were gradually replaced by young mice from the reserve pool so that all mice in the room were approximately the same age. This process started the last week in January, 1948, and the room was finally completed the third week in March. As a result of these procedures, room 2 was never completely evacuated, and some infected mice remained there at all times.

Room 3, in which the epidemic had been mild, on the other hand, was completely evacuated of all mice, thoroughly cleaned, and disinfected before any mice were returned to it. The mice placed on the control shelf, group E, were some of those removed from room 2 during the "setting" of that room. They were placed in room 3 the second week in February and the room was completely "set" the third week in March. Each room had a separate attendant who was not permitted to enter any other room. This room then was completely cleaned and free of mice prior to the experimental arrangement.

Presence of the disease in both rooms was determined by fecal cultures from the control boxes, and heart blood and spleen cultures from randomly selected mice. Throughout the experiment, random cultures were taken from time to time to be sure that the disease was present, especially in room 3. Each group of cultures from the control mice of both rooms showed positives. For all mice in room 2 this condition persisted to the end of the experiment, but in room 3, as the work progressed, the number of positives in each lot of cultures gradually diminished; and after the end of April, nearly all of the culture groups from the treated animals were entirely negative.

The records were cards on which were recorded the total number of dead original mice "set up," the number of replacement mice introduced into the boxes, and the number of these which died. There was also a column for the number of mice which were missing from the boxes (possible escapes). These last proved to be so few that they were included in the final totals as deaths. The sexes were accounted for separately. All recording was by calendar weeks. Separate cards were kept for each block of ten boxes (60 mice) so that position effects, if any, in the rooms might be noted. At the close of the experiment, the records were analyzed statistically.

EXPERIMENTAL RESULTS

No position effects were noticed except at the start while the rooms were being set. In room 2 it was noted that deaths were very few in newly set blocks for the period preceding the first parturitions (three to four weeks). At this time, deaths occurred in large numbers in both groups, and this wave of deaths followed the blocks of mice moved into the room. The female death percentage greatly exceeded the percentage of male deaths in this first wave. After the entire room had passed this period, there was no significant difference between any of the blocks, or combination of blocks nor between the sexes. In room 3, where the death rates were low, no position effects were noted, and there was no evident increase in the number of deaths during the period of first parturitions. The data presented on death rates start at the time when all the animals in the rooms had passed this period.

An unexpected result of the treatment was the great improvement in the appearance and viability after weaning of the young mice produced by the vaccinated breeders. This was true of both rooms.

The total death rates for room 2 from April 5 to June 28th are presented in table 1. The death rate expressed as a percentage, essentially the ratio of deaths within the week and the number alive at the beginning of the week, is shown graphically to July 19th in figure 1. No significant difference was noted in the trend of the data up to June 28th between the vaccinated and the unvaccinated animals whether analyzed by chi square analysis or in terms of t ratio. After that date the curves diverge sharply, indicating a differential death rate between the two groups. During the period following June 28th however, the room was being "taken down," and during the weeks beginning July 5 and 12th, 19 original unvaccinated animals out of a total of 182

TABLE 1.—*Comparisons of deaths in room 2.*

Comparison	Group	Period	Survivors	Died	Total	Percent mortality	Chi-square
1	A. Unvacc. original	4–5 6–28	182	538	720	75	281.94*
	B. Vacc. replace.	4–5 6–28	451	186	637	29	
2	A. Unvacc. original	4–5 6–28	182	538	720	75	1.15†
	C. Vacc. original	4–5 6–28	586	1574	2160	73	
3	C. Vacc. original	4–5 6–28	586	1574	2160	73	103.73*
	D. Vacc. replace.	4–5 6–28	761	1025	1786	57.5	
4	B. Vacc. replace.	4–5 6–28	451	186	637	29	3.42†
	D. Vacc. replace.	4–5 6–28	761	1025	1786	57.5	

* Exceeds the 0.1% level of significance.
† Not significant.

were removed from the room. During the week of July 19th, 34 out of 76 unvaccinated and 27 out of 509 original vaccinated animals were removed. It will be seen that the trends of the curves had been established by these dates.

The death rates for the replacements in both groups, if the series are compared from their respective origins, are very similar (fig. 2). An interesting comparison is that between the unvaccinated originals and the vaccinated

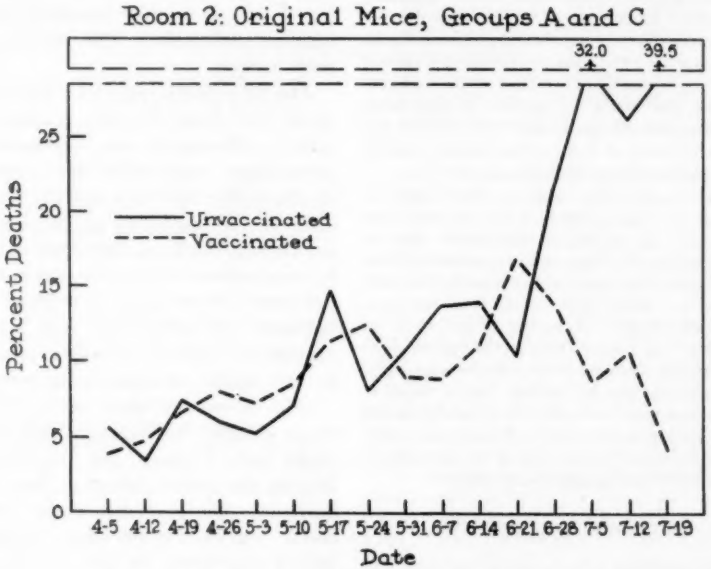

FIG. 1.—Deaths of original mice, groups A and C; room 2.

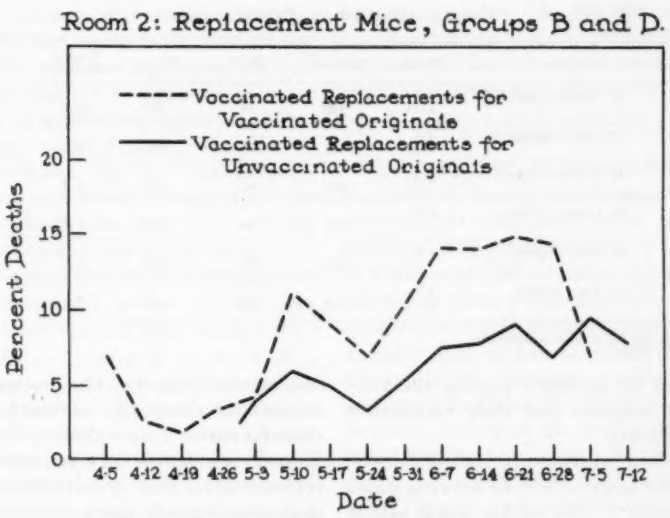

FIG. 2.—Deaths of replacement mice, groups B and D; room 2.

replacements for this group. The death rate in the former is consistently higher and rises sharply (fig. 3) after June 21st, while the rate for the latter remains rather constant and significantly lower. If these death rates from week to week are considered as independent observations, the application of the t test to the pairings of nonvaccinated originals and vaccinated replacements yields a significant difference in favor of the vaccination. No such significant difference is

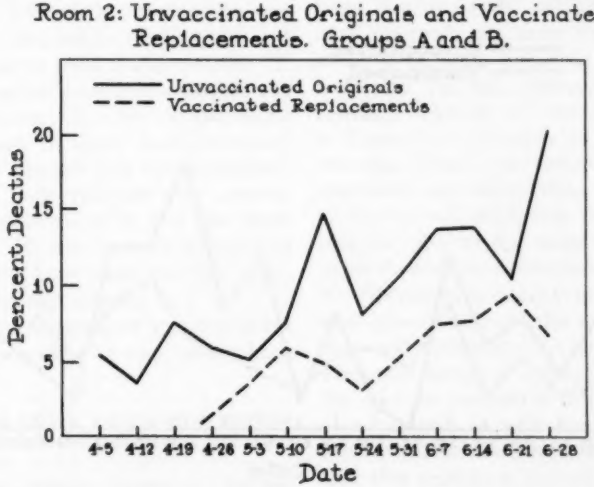

FIG. 3.—Deaths of unvaccinated originals and vaccinated replacements, groups A and B; room 2.

TABLE 2.—*Comparisons of deaths in room 3.*

Comparison	Group	Period	Survivors	Died	Total	Percent mortality	Chi-square
1	E. Unvacc. original	4–5 8–23	598	74	672	11.0	4.74*
	G. Vacc. original	4–5 8–23	1914	172	2086	8.2	
2	E. Unvacc. original	4–5 7–12	612	60	672	8.9	2≡0
	F. Unvacc. replace.	4–5 7–12	103	10	113	8.85	
3	G. Vacc. original	4–5 7–12	1960	126	2086	6.0	5.39*
	H. Vacc. replace.	4–5 7–12	180	4	184	2.2	

* Exceeds the 5% level of significance.

obtained by similarly pairing the vaccinated originals and their vaccinated replacements.

The data presented in table 2 demonstrate the large difference between room 3 and room 2. The weekly death rate is consistently below 1.5% in room 3 for the original groups, and the difference between the vaccinated and unvaccinated mice is statistically significant (chi square or *t* test analysis) in favor of vaccination (fig. 4). The bacterin decreased the death rate although conditions for survival were good in the room. Since so few deaths occurred among the replacements, that one death may be disproportionately large, statistical analysis was not undertaken. It was noted, however, that the unvaccinated replacements, group F, died more frequently than the vaccinated ones, group H, 8.85 and 2.2%, respectively.

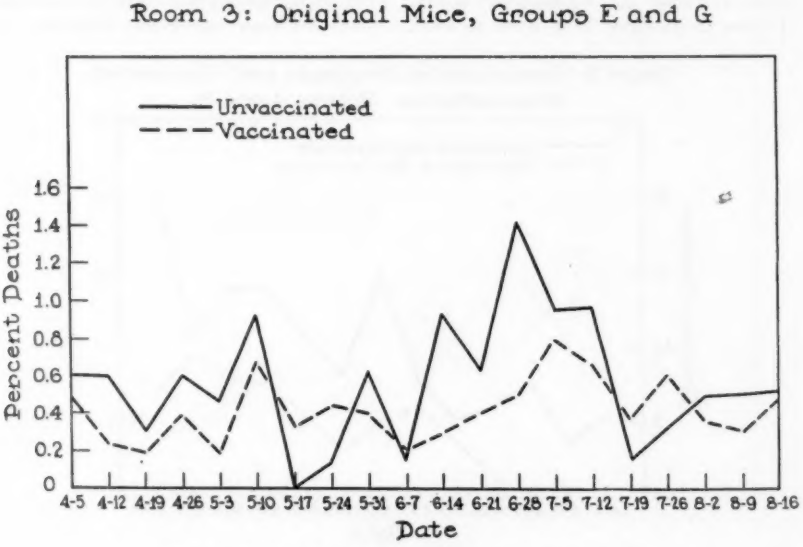

FIG. 4.—Deaths of original mice, groups E and G; room 3.

A comparison of the death rates between the two rooms indicates that the method of withdrawing young mice at weaning time to a separate room is efficient in preventing spread of infection, since all mice used in both rooms were produced by the unvaccinated mice in room 2, which had an appallingly large death rate, and were selected at random for return to the rooms. The death rates in the unvaccinated groups for a comparable period of time (ten weeks) were: room 2, 58%, room 3, 4%.

During the course of the work with TCL two other vaccines were tested in a limited number of animals. One was a bacterin (TE),* made with phenol-killed organisms of Salmonella Center culture #4185 of *S. enteriditis*, the organism infecting the mouse colony, and culture #3703, *S. typhimurium*, derived from pigeons. This was prepared to contain 1000 million of each of the two organisms per ml. The dosage was 1/10 ml of a 1:1000 dilution, and two intraperitoneal doses were given one week apart. The second was a Bacterial Antigenic Complex (BAC)† of the same organisms. The titer of this preparation was computed on a total nitrogen basis, and a dose equal in total nitrogen to the TE dose was given intraperitoneally on the same schedule. Because of the small groups used in these tests, complete statistical analysis was not attempted. However, both vaccines were consistently better than TCL, and the death rate when TE was used was slightly and consistently lower than the BAC rate, though not significantly so.

Since the experiment was concluded all CFW breeders in our colony have had two doses of TE, 500,000 of each organism to the dose. The death rate has been consistently at the level of the room 3 experimental mice.

* Supplied through the courtesy of American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y.

† Supplied through courtesy of Hoffman Laboratory, Paterson, N. J.

DISCUSSION

The experiments of Topley and others (reviewed in Topley and Wilson, 1946) showed that mice immunized against salmonella by intraperitoneal infection of a bacterin killed by formalin and heat did not produce a high degree of resistance to the intraperitoneal infection of a lethal dose of live salmonellae. Our findings in this case were identical using a phenol-killed bacterin. Webster (1937) however, informed us that phenol or heat-killed bacterin gave a much higher degree of immunity than formalin-killed. Topley found that for immunized animals introduced into a group of non-immunized only a short extension of life had been produced by the immunization. The graphs shown in figure 3 indicate that this was not the case with the particular strain of mice with which we were working. According to Gowen (1950), the heredity of the mice has a large bearing on the amount of resistance attained; the more resistant the strain is to begin with, the higher the immunity reached. Perhaps the discrepancy between our results and that of Topley is explainable on this basis, and that Topley was working with an extremely susceptible group. The height of the over-all death rate in room 2 is probably due to the massive infection to which these mice were subjected, and the differences in death rates in room 3 more closely indicate the true measure of the effectiveness of the immunization where the dosage of pathogens to which the mice are exposed is small. An incident occuring in room 3 a year after experiments where completed indicates that this opinion is correct. A box appeared in which every mouse had severe

typhoid. The week following its removal, the disease appeared in four adjoining boxes. Following removal of those, no further cases have been observed and fecal tests have been negative. Invariably prior to our use of immunization, when mouse typhoid had started to travel from box to box, an epidemic, mild or severe, followed.

CONCLUSIONS

1. Room differences and all that these connote are highly significant as regards death rates in a colony suffering from an epizootic.

2. Despite the minimal dosage of the vaccines administered, beneficial effects are noted in that the death rates were reduced.

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THERAPY OF EXPERIMENTAL BRUCELLOSIS

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The effective treatment of human brucellosis remains an unsolved problem. The disease is difficult to diagnose, characterized by spontaneous remissions and, while not rare, is usually of such limited occurrence as to preclude the inclusion of controls in any therapeutic trial. These circumstances have led to the development of a considerable body of literature claiming therapeutic efficacy for many forms of treatment which have later proved ineffective. Eisele (1950) has ably summarized the literature, concluding that treatment with a combination of sulfadiazine and streptomycin offers the greatest promise. Eisele and McCullough (1947) have set forth criteria for choosing cases of brucellosis for evaluation of therapeutic measures which most of the published studies of clinical use of therapeutic agents regrettably fail to meet. It seems fair to observe that, except in very large groups of cases, failure of treatment is more significant than success; since the majority of brucellosis patients recover completely with only supportive treatment, failure of a drug to terminate the disease in a few cases despite apparent success in others should give rise to skepticism.

In view of these difficulties, recourse must be had either to in vitro tests or to animal experiments. The former seem of little value because of the number of agents to which brucellae are sensitive in the test tube but which have proven to be of little value in treatment of the disease. Testing in experimental animals appears to offer more promise but here again the problems are manifold. The large numbers of animals to be housed for long periods strain the facilities of the ordinary laboratory. Secondly, the criteria to be adopted for success or failure of treatment are difficult to formulate. In the present work, inability to recover the invading organisms was selected as the criterion of cure. The validity of this standard will be discussed below, but it may be noted here that the absence of organisms is not easy to establish with certainty: the selection and treatment of tissues to be cultured and the medium and conditions of cultivation may all affect the results. The time between the end of treatment and sacrifice may also influence the outcome.

These considerations may explain certain discrepancies which have appeared in the literature, although in some cases dosage levels, route and time of administration of the therapeutic agent, strain, virulence and dose of the infecting organism, and susceptibility of the host animal may also bear upon the results. Thus Live et al (1946) found streptomycin to be of value in reducing the percentage of infected guinea pigs yielding *Brucella abortus* after treatment, whereas Holm and McNutt (1949) found no effect from this antibiotic. The guinea pigs used by the latter investigators were infected with larger numbers of organisms and treated with smaller doses of antibiotic. Working with *Brucella suis*, Kelly and

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Henley (1947) and Carle and Larson (1950) found streptomycin to be without effect. Similarly, Heilman (1949) found combined aureomycin-dihydrostreptomycin treatment more effective than streptomycin-sulfadiazine in reducing the number of organisms in the spleens of mice infected with *Br. abortus* or *suis*. Holm and Moore (1950), studying the same combinations in *Br. abortus* infection of guinea pigs, found the streptomycin-sulfadiazine combination more effective. This type of contradiction is found throughout the literature on experimental brucellosis therapy. It is probably inevitable, as each group of workers must choose experimental conditions which in their judgment seem most suitable, but it renders even more difficult the extrapolation from experimental animal to human.

The guinea pig is the animal of choice for therapeutic studies in the opinion of most investigators because the human disease is more closely simulated in this species than in any other readily available animal. The relatively large quantities of drugs and amount of space required for this host have, however, stimulated a search for a suitable smaller animal, and the selective toxicity for guinea pigs of many antibiotics (penicillin, aureomycin, Chloromycetin) may make this of more urgency. In the work to be reported, representative experiments using embryonated eggs or white mice are cited, but for the bulk of the work guinea pigs were employed.

METHODS

Mediums

Tryptose agar (Difco) prepared as directed or modified by the addition of dextrose, $FeSO_4$, and thiamin (McCullough et al, 1947) was used throughout for the cultivation and isolation of the organism. Tryptose saline (0.1% Bacto tryptose and 0.5% NaCl) was used as the suspending and diluting fluid.

Strains of brucella

The organism used for infecting experimental animals was *Br. suis*. The first strain used, designated S1, was a subculture of a strain furnished through the courtesy of Dr. I. F. Huddleson of Michigan State University, and labelled by him 1772-A. Its virulence declined after several years, and strain PSIII, obtained through the kindness of Dr. L. M. Hutchings of Purdue University, was substituted. This strain or a smooth clone derived from it (PSIIIk) was used in the remainder of the work. All strains were maintained on tryptose agar with frequent transfer of S1 and infrequent transfer of PSIII.

Strains and maintenance of host animals

White Swiss mice from a colony originally derived from a Webster strain were used. They were kept on wood shavings in Monel metal cages and fed commercial mouse diet and tap water ad libitum. The mice weighed 16 to 20 g at the start of the experiments. Except where specified, guinea pigs were of mixed stock. In general, they weighed 350 to 450 g at the start of the experiments. They were maintained in cages similar to those used for mice and were fed commercial rabbit diet, carrots, and either green food or water containing ascorbic acid.

Preparation of infecting doses

The growth from a 24- or 48-hour agar slant was suspended in tryptose saline and diluted in the same menstruum to yield the desired dose in 0.1 or 0.2 ml. Pour plates were made of appropriate dilutions in order to determine the actual dose given. The organisms were injected subcutaneously in the right groin except that in a few of the guinea pig experiments the intracutaneous route was used.

Autopsy technique

a. When serologic studies were to be done, the animals were bled under chloroform anesthesia. They were then sacrificed, portions of the spleen, liver, and the precrural lymph node were removed aseptically and triturated in tryptose saline, and aliquots of the resulting suspension were spread on the surface of agar plates. These were incubated for 72 to 96 hours at 37 C and examined for the presence of *Br. suis* colonies. Doubtful colonies were checked by the slide agglutination technique.

b. Agglutinin titers were determined in the serum by means of a tube agglutination test using the Army Medical School tube antigen.

Administration of therapeutic agents

a. *Sulfadiazine* was administered mixed with the food. For treatment of mice, the mouse pellets were ground and mixed with 0.5% (by weight) sodium sulfadiazine, then moistened with water and refashioned into cakes. This procedure was impracticable for the large quantities of food required for guinea pig experiments. For this purpose, a suspension of sulfadiazine in 95% alcohol was sprayed onto rabbit pellets in sufficient quantity to yield a final concentration of 2% (by weight) of the food. At the start of treatment in all experiments, the animals were given one or two subcutaneous injections totalling 75 to 150 mg of sodium sulfadiazine per guinea pig or 5 mg per mouse in order to raise the blood level quickly.

b. *Streptomycin sulfate* (Pfizer) was dissolved in sterile distilled water and administered subcutaneously. The daily dose for mice (1200 μg/mouse/day, approximately 60 mg/kg) was divided into eight portions given at three-hour intervals. Guinea pigs received the antibiotic at intervals of three, four, or six hours; the dosage level was high, starting in most experiments at 160 to 180 mg/kg/day, which, except in the last experiment, was cut to 120 to 140 mg/kg/day after about 10 days of treatment.

c. *Chloromycetin* (Parke, Davis & Co.) was given either by forced feeding or by simultaneous subcutaneous and oral administration. For forced feeding the drug was suspended in propylene glycol and administered by means of a hypodermic syringe attached to a blunt needle which was inserted into the esophagus. The dosage was approximately 270 mg/kg/day in four doses daily. For subcutaneous injection the antibiotic was suspended in sterile 10% gum arabic, and a total of 50 mg/kg/day were administered in four doses daily. In addition, the animals ate ad libitum pellets containing 1% (by weight) Chloromycetin. The feed was prepared by suspending the antibiotic in a 35% aqueous solution of dextrose and moistening the pellets with the mixture (Feldman and Hinshaw, 1945).

d. *Aureomycin* (Lederle intravenous) was given either intraperitoneally or by the forced feeding method described for Chloromycetin. For intraperitoneal administration the drug was dissolved in the leucine diluent supplied by the manufacturer until it became obvious that the animals were in great discomfort after injection. A phosphate buffer, pH 7, was therefore substituted for the highly alkaline leucine solution. The volume administered per dose was simultaneously increased from 0.2 to 0.5 ml without changing the quantity of drug administered (20 mg/kg/day). The phosphate buffer was also used in preparing the solutions for forced feeding. The same dosage was used, but it was given in only two doses per day instead of four, as in the animals treated intraperitoneally.

e. *Terramycin hydrochloride* (Chas. Pfizer & Co.) was dissolved in distilled water, neutralized with NaOH, and administered subcutaneously in two doses daily of 0.2 ml each. Details of dosage are given in the description of the experiment in which it was used.

f. *Neomycin sulfate* (Upjohn Co.) was dissolved in sterile distilled water and administered subcutaneously in three doses per day totalling 50 mg/kg.

g. *Immune serum* (Sharpe and Dohme Lyovac Antibrucella serum) was reconstituted with sterile distilled water and administered intraperitoneally.

Drug screening in embryonated eggs

Nine- to 11-day embryos were infected via the yolk sac with 1×10^3 to 1×10^5 organisms of *Br. suis* S1. This sufficed to kill 50% of untreated embryos within two to four days. The drugs were usually given just after infection or mixed with the organisms immediately before injection.

RESULTS

Attempts were made to assay drugs by methods other than the guinea pig test, and a few of these will be reported. The in vitro test was soon abandoned in favor of in vivo methods because most of the commonly employed antibacterial agents inhibited the growth of *Br. suis* in rather low concentrations but proved ineffective in vivo. In all of the investigations in vivo an attempt was made to favor the drugs, in order that the potentialities of any therapeutic agent might not be overlooked. This was accomplished by the use of small numbers of organisms to infect the host and by early treatment with rather high doses of the drug under test.

In an effort to develop a test which would be more selective than the test tube method yet more rapid and economical than the guinea pig test, the embryonated hen's egg, of which others (Jones et al, 1944; Metzger et al, 1942;

TABLE 1.—*The effect of streptomycin on murine brucellosis.*

Treatment	Mice sacrificed at end of therapy		Mice sacrificed 2 weeks after end of therapy	
	Infection ratio*	Percent positive	Infection ratio	Percent positive
Streptomycin† 0 hr.	7/50	14	17/26	61
Controls	27/43	63	13/40	33
Streptomycin† 48 hr.	23/34	68	9/35	26
Controls	10/20	50	7/29	24

* Positive cultures/total cultured.
† 1200 μg/mouse/day in 8 doses at 3-hour intervals.

Shaffer and Spink, 1948) have made use for screening of antibrucellar drugs, was the first system used. Under the conditions of the test most therapeutic agents used (sulfonamides, streptomycin, atabrine, promine) prolonged the life of the embryos to hatching time. In some cases administration of drug was withheld up to 48 hours after infection. This decreased the sensitivity of the test considerably, and it is possible that by such adjustment of the conditions a satisfactory screening system could be developed. Results were not, however, readily reproducible, and further work would be required before the test could be designed to give results compatible with those of other testing methods.

Infections in white mice were also employed as alternative to the guinea pig method. The doses of organisms required to produce 100% infection of untreated controls in these animals were so large as to constitute too severe a test for the drugs (Spencer and Kelly, unpublished). A transitory infection could, however, be established in 80 to 90% of the animals by the use of moderate infecting doses (approximately 1×10^5 organisms of the *Br. suis* S1 strain). This is useful for the testing of drugs such as penicillin, to which guinea pigs are intolerant, and has the advantage of requiring relatively small quantities of drug. The experiment to be presented is an example of the use of this technique.

Streptomycin in murine brucellosis

In a test of the efficacy of streptomycin, four groups of mice were infected with about 1×10^5 organisms of the S1 strain of *Br. suis*. One group was given streptomycin immediately after infection, while treatment was started in another group 48 hours after infection. Treatment was continued for ten days. The two remaining groups served as controls. Twenty-four hours after the end of treatment one-half of the animals were sacrificed and cultures made of the spleens. The remaining animals were sacrificed 14 days after the end of treatment. The results are summarized in table 1.

In the animals left untreated for 48 hours the drug had no effect. In the mice treated immediately, the infection was suppressed but within two weeks had begun to assert itself, while the untreated control mice had begun to recover.

Therapy of cavine brucellosis

Because of the many claims (Pulaski and Amspacher, 1947; Eisele and McCullough, 1947; Spink et al, 1947 and 1949) for sulfadiazine and streptomycin, alone or in combination, as effective agents for the treatment of human brucellosis, the bulk of the work in guinea pigs to be reported concerns these two compounds. As the newer antibiotics have become available they have also been tested, although some of these are, like penicillin, toxic for guinea pigs, which made the tests unsatisfactory. Some work on immune serum is also included. In all experiments treatment was started within three weeks after infection and, in most cases, continued for two weeks. When possible, one-half of each treated group was sacrificed at the end of treatment, and the remainder one month later, in order to detect the occurrence and the permanence of any

TABLE 2.—*Effect of sulfadiazine and immune serum on cavine brucellosis.*

Therapeutic agent†	Postinfective day treatment started	Infection ratios* at time of sacrifice			
		1 week§		4 weeks§	
			Percent		Percent
Sulfadiazine	0	0/14	0	15/15	100
	8	1/19‡	5	8/20	40
	11	4/20	25	13/21	62
	15	1/20‡	5	7/18	39
Immune serum	11	15/15	100	16/16	100
Sulfadiazine and serum	11	5/19	26	9/20	45
None		42/42	100	36/41	88

* Number infected/number cultured.
† Administered for 30 days.
‡ Lymph nodes not cultured.
§ After end of treatment.

tendency of the drug to arrest the course of the disease. In several experiments, intercurrent infections in the animal colony made it necessary to sacrifice the animals at the end of treatment.

Sulfadiazine, streptomycin, and immune serum in cavine brucellosis

The protocols and conditions of the individual experiments differed so little that the results of each type of treatment can be pooled. Sulfadiazine was administered for 30 days in some experiments and 14 days in others, and since the effects of the two forms of treatment differed considerably, they are presented separately.

In the two experiments, sulfadiazine was administered for 30 days; in the first, treatment started at 0, 8, or 15 days after infection, in the second at 11 days. The second experiment included two additional groups, one treated with immune serum and one with both serum and sulfadiazine. The results are set forth in table 2, from which it is apparent that treatment with sulfadiazine early in the course of the disease succeeded in suppressing detectable evidence of brucellosis, and this was confirmed by the gross pathologic appearance of the tissues at autopsy, but within a few weeks after the end of treatment the disease became evident. Delaying treatment for several days, on the other hand, resulted in a lower percentage of "relapses" after treatment. Immune serum, which was used in one experiment, proved ineffective by itself but slightly decreased the incidence of "relapse" when added to sulfadiazine treatment.

In the second of these experiments, five animals from the sulfadiazine-treated group and five control animals were sacrificed every five days during the period of therapy to obtain data on the progress of the infection under the influence of sulfadiazine. The findings are presented in table 3, which shows that the greatest effect of sulfadiazine as indicated by cultural and serological studies occurred during the first 15 days of therapy. The rapid reduction of recoverable organisms in the spleen and liver under sulfadiazine treatment is of interest. In only one of the 15 animals

TABLE 3.—*Progress of* Br. suis *infection in guinea pigs during treatment with sulfadiazine.*

Time after infection (days)	Days treated*	Positive cultures†			Positive agglutinin titer‡
		Lymph node	Spleen	Liver	
8	0	4/4	3/5	2/2	0/4
16	0	5/5	5/5	5/5	3/5
16	5	5/5	5/5	4/5	0/5
21	0	5/5	5/5	2/2	5/5
21	10	2/4	0/5	0/4	2/5
26	0	5/5	5/5	5/5	5/5
26	15	3/5	1/5	0/5	0/5
32	0	3/3	5/5	4/5	5/5
32	21	5/5	0/5	0/5	0/5

* Animals treated from 11th postinfective day.
† Number positive/total cultured.
‡ Agglutination at a dilution of at least 1:100.

sacrificed at ten or more days of therapy could organisms be isolated from these organs, although *Br. suis* persisted in the lymph nodes through 21 days of treatment.

The results of five experiments in which streptomycin, sulfadiazine, and immune serum were used alone or in combination are presented in table 4. Sulfadiazine was given for only 14 days in an attempt to reduce its effectiveness so that additive or synergic effect of the with the combination) yielded positive cultures. This group is not represented in table 4 but is noteworthy because it indicates that the interval between infection and autopsy usually used for detecting "relapses" (four weeks) may have been insufficient.

Other antibiotics

In two of the experiments represented in table 4, Chloromycetin (forced feeding or by the subcutaneous and oral

TABLE 4.—*Effect of sulfadiazine, streptomycin, and immune serum on cavine brucellosis.*

Therapeutic agent‡	Infection ratios§							
	Early treatment*				Late treatment*			
	Early Sacrifice†		Late Sacrifice†		Early Sacrifice†		Late Sacrifice†	
		Percent		Percent		Percent		Percent
Streptomycin	28/30	93	21/21	100	21/23	91	28/30	93
Serum	18/20	90	10/10	100				
Sulfadiazine	25/31	81	24/26	92	13/19	68	19/23	83
Sulfadiazine and streptomycin	18/39	46	7/22	32	15/31	48	16/43	37
Sulfadiazine and serum	16/20	80	10/10	100				
None	29/32	91	19/19	100	43/43	100	30/39	77

* Early treatment started 10 to 12 days after infection; late treatment started 14 to 21 days after infection.
† Early sacrifice = less than 1 week after treatment; late sacrifice = 2 to 4 weeks after treatment.
‡ Administered for 14 days.
§ Number positive/total cultured.

other agents might be detected. It is apparent that under these conditions sulfadiazine alone was little more effective than streptomycin. It may be seen that, like serum, streptomycin alone in the large doses used did not noticeably affect the course of the disease. Addition of serum treatment to sulfadiazine did not improve the results, but streptomycin combined with sulfadiazine "cured" a fairly large proportion (65%) of the animals. In animals treated with this combination neither the time of initiation of treatment (ten to 21 days) nor the time of sacrifice after treatment (three to 31 days) significantly affected the outcome of the experiments.

In one of these experiments, ten each of the animals treated with sulfadiazine alone or in combination with streptomycin were not sacrificed until seven weeks after the end of treatment. Of these 20 animals all but one (treated routes) was included, either alone or combined with sulfadiazine. A number of guinea pigs died, apparently as the result of the treatment, but in the survivors the antibiotic appeared to have had no beneficial effect. Aureomycin administered intraperitoneally was used in the second of these experiments, but as used it killed the majority of the animals.

The final experiment (table 5) included tests of the efficacy of lower doses of streptomycin (Holm and McNutt, 1949) and of the effect of adding aureomycin to the sulfadiazine-streptomycin treatment, and in the same experiment terramycin and neomycin were given preliminary trials. This experiment is recorded separately because of the complexity of the protocol, but it may be noted that the animals treated with streptomycin and sulfadiazine, alone or together, are also included in table 4.

Guinea pigs of the "Hartley" strain were infected subcutaneously with 65 organisms of the PSIIIk strain of *Br. suis*. The animals were to have been treated from the 14th to the 28th post-infective days with various drugs as shown in table 5. Terramycin was so poorly tolerated that the dosage (initially equivalent on a weight basis to a high human dose) was reduced on the fifth day and treatment with this drug was discontinued after ten days. In most groups one-half the animals was to be sacrificed at the end of treatment, the remainder one month later. On the basis of previous experience this was considered unnecessary for group 5, and there was reason (Holm and McNutt, 1949) to believe that in group 6 the number of animals yielding positive cultures would be so few that any difference between this group and group 7 would not be detectable. No animals of groups 5 and 7 and the aureomycin control group 8, therefore, were included for sacrifice at the end of treatment. Unfortunately, most of the aureomycin controls died of intercurrent infection, and only two remained for sacrifice at the alloted time.

It is apparent from table 5 that of the treatments tested, the combination of streptomycin, aureomycin, and sulfadiazine was the most effective. No animals receiving this treatment showed evidence of active brucellosis when examined one month after the end of treatment. The combination of terramycin and sulfadiazine appeared to arrest the disease temporarily, reducing the proportion of animals positive for brucellosis to 15% at the end of treatment. Within the succeeding month, however, this percentage rose to 80. In varying degrees this phenomenon occurred also in groups 1, 4, and 9. Group 2, receiving the low dose of terramycin alone, showed a high (75%) infection rate at the end of treatment, which appeared to decline during the subsequent holding period. Further investigation of this observation would be desirable. Whether the combination of aureomycin and streptomycin without sulfadiazine would be as effective as the use of all three remains to be determined.

TABLE 5.—*Effects of various antibiotics, alone and in combination, on* B. suis *infection in guinea pigs.*

Group	Number of animals	Drug and dosage/day*	Treatment days (after infection)	Infection ratios‡				Deaths during treatment
				31st day¶		59th day¶		
					Percent		Percent	
1	29	Terramycin, 20 mg‖	14–24	5/12	42	8/12	67	5
2	29	Terramycin, 10 mg†	14–24	9/12	75	7/14	50	3
3	30	Terramycin, 10 mg†+sulfadiazine	14–24§	2/13	15	12/15	80	2
4	20	Sulfadiazine, 2% of food	14–28	4/10	40	7/10	70	0
5	14	Streptomycin, 160 mg+sulfadiazine	14–28			4/14	29	0
6	29	Streptomycin, 40 mg+sulfadiazine	14–28	3/14	21	7/15	53	0
7	20	Streptomycin, 40 mg+aureomycin+sulfadiazine	14–28			0/13	0	7
8	10	Aureomycin, 20 mg	14–28			0/2	0	8
9	20	Neomycin, 50 mg	14–28	5/10	50	9/9	100	1
10	20	Untreated controls		10/10	100	9/10	90	1

* "Mg" refers to mg/kg/day.
† Reduced to 5 mg on 5th day of treatment.
‡ Number positive/total cultured.
§ Sulfadiazine continued to 28th day.
‖ Reduced to 10 mg on 5th day of treatment and to 5 mg on 9th day.
¶ After infection.

DISCUSSION

The results of the guinea pig tests show that combined antibiotic and sulfonamide therapy is of value in the treatment of cavine brucellosis, as in-

dicated in the literature in the case of the human disease. To that extent, these studies indicate that the experimental disease in guinea pigs may serve as a model for study of human brucellosis. That important differences between the two diseases exist is, however, also suggested. The high incidence of "cures" (guinea pigs yielding negative cultures) resulting from 30-day sulfadiazine treatment has not been obtained by similar treatment of humans (Howe et al, 1947).

Lack of information on the nature of these differences complicates selection of suitable conditions for testing and the formulation of criteria for success or failure of treatment. Even if these questions could be satisfactorily answered, direct application of experimental results to human treatment would remain difficult. Such factors as the relative native resistance of humans and of guinea pigs, their differential ability to develop immunity during the course of the disease, and the influence on the outcome of the human disease of previous exposure to subinfective doses of brucella might still cause differences in the response of the two species.

It is possible that the criterion used in this work (lack of recoverable organisms in the tissues) is more rigorous than necessary. Humans can probably return to a state of apparent health with no objective or subjective signs of infection before the organisms have been completely eliminated from their tissues; this is indicated by the periods of well-being which often precede clinical relapse as well as by recent studies indicating that humans who have recovered from brucellosis may carry brucella organisms with no sign of ill health (McVay et al, 1950). Absence of recoverable organisms was, however, chosen for this work because it is possible that as long as the pathogen is present, the danger of relapse exists. On the other hand, our criterion for relapse may not have been valid. The instance cited in which animals sacrificed seven weeks after therapy showed a high incidence of infection may indicate that a period of four weeks after the end of treatment was insufficient to detect relapses.

These results have shown that the interval between infection and administration of the therapeutic agent may influence the outcome in guinea pigs and mice (tables 1 and 3). In the case of treatment with streptomycin-sulfadiazine this interval appeared to have no influence, but this may be due to the fact that the combined treatment was never used before the tenth postinfection day. It would seem reasonable to suppose that the ability of the host to develop immunity may be a critical factor in this connection. A therapeutic agent administered too early may fail to eliminate the pathogen while suppressing the infection sufficiently to prevent a reaction by the host. The remaining organisms may thus be able to multiply freely and establish disease after cessation of therapy. Withholding of treatment for too long may, on the other hand, result in failure of the drugs to reach organisms which may be present intracellularly or in walled off abscesses. The ideal time for initiation of treatment would undoubtedly vary among individuals, depending on their innate resistance, the size of the infecting dose, and the route of entry. These and other factors would likewise affect the ideal duration of therapy. Means for enhancing the development of immunity in the host might be a useful adjunct to treatment. The intermittent treatment method which has recently been suggested may have the same effect by permitting repeated antigenic stimulus, though it, of course, involves unnecessary inconvenience to those patients who would not have relapsed anyway and might result in selection of

drug-resistant strains.

The embryonated egg and the mouse may prove satisfactory for drug screening when more data are available on their correlation with clinical results. Selection of a suitable strain, age, and weight range of the mouse and an appropriate strain and dosage of infecting organism would undoubtedly improve the reliability of the tests. Until such data are available, the high natural resistance of mice and susceptibility of chick embryos render them suspect as hosts for model infections and the guinea pig must remain the small animal of choice.

It seems established that sulfonamide-antibiotic therapy can terminate acute episodes in human brucellosis. Relapses do occur, however, and continued investigation of modified or alternative treatment is needed. While a new drug may be found capable of effecting complete cure, an attempt to influence the host response might be more immediately rewarding than continued screening of antibiotics. For this purpose research in the therapy of experimental brucellosis might profitably be directed toward the problems of standardizing the guinea pig test. Adjustment of experimental conditions (e.g., virulence of infecting organism, time of sacrifice after the end of treatment) with a view to reproducing but not exceeding the partial success obtained in human treatment is a first essential, as these experiments have shown. The achievement of such standardization would permit more intelligent investigation of additions or alternatives to the "standard" treatment. Methods for increasing the immunity, the cellular response, or the hormone levels of the host would seem to be fruitful lines of approach.

SUMMARY

The combined use of streptomycin, sulfadiazine, and aureomycin proved the most efficacious of several treatments in guinea pigs infected with *Brucella suis*.

Difficulties in the way of applying results in guinea pigs to human infection are discussed.

The embryonated hen's egg and the white mouse, under the conditions used, proved to be unsatisfactory hosts for experimental therapeutic studies.

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THE EMBRYONATED EGG AS A CULTURE MEDIUM FOR THE ANIMAL PHASE OF *COCCIDIOIDES IMMITIS*

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Coccidioides immitis occurs in two different forms, depending upon its environment. In the animal, including man, this organism exists in the form of thick-walled spherules ranging in sizes from 2 to 120 μ in diameter and characteristically reproduces by endosporulation. This form is generally referred to as the animal phase or parasitic stage of growth. The individual organisms are referred to as sporangia or spherules. In or on artificial mediums, *C. immitis* exists in the so-called vegetative or mycelial phase of growth, which is characterized by the development of a mycelium composed of hyphal elements. Reproduction in this phase is accomplished by the development of branching filaments and arthrospores.

In the laboratory investigation of *C. immitis*, it is highly desirable to study the animal phase of this organism, but to date there has been no in vitro method developed whereby this organism grows exclusively in the animal phase. Several laboratory animals have been used for the growth of this phase of *C. immitis* but it is difficult to isolate the organism for study. Recently several preliminary reports in the literature have indicated that the embryonated chicken egg was suitable for the development and growth of large numbers of spherules of *C. immitis*.

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In 1941, Moore[1] used the chorioallantoic membrane of the developing chick embryo as a medium for the cultivation and histopathologic study of pathogenic fungi. In his evaluation of the growth of *C. immitis*, inoculations were made onto the chorioallantoic membrane of the egg. They were incubated at 33 C in a regular bacteriological incubator and were observed seven days after inoculation. Microscopically he observed a translucent, granular exudate which appeared in layers or striations on the ectodermal surface of the chorioallantoic membrane. This contained *C. immitis* in various stages of development, with the formation of characteristic endosporulating cells of the parasitic stage of the fungus. The inoculated filaments were first converted into arthrospores and then into sphericle cells which enlarged and developed endospores. In seven days on the chick membrane the organism had shown a reversion to its parasitic role. Two photographs of the animal phase of *C. immitis* within the translucent, granular exudate were presented. It was his feeling that this method held great investigative possibilities.

In 1950, Burke[2] extended the evaluation of the culture of the animal phase of *C. immitis* in the embryonated egg not only in the chorioallantoic membrane but to include the embryo per se,

1. Moore, M. 1941, The chorio-allantoic membrane of the developing chick as a medium for the cultivation and histopathologic study of pathogenic fungi. Am. J. Path. **17**: 103–120.
2. Burke, R. C. 1950, Coccidioidomycosis. Tr. New York Acad. Sc. **12**(2): 188–194.

allantoic fluids, air sac membrane, albumen, etc. It was her opinion that the sporangial stage reproduced in fertile chicken eggs in massive numbers and that the results were so spectacular that the use of the fertile chicken egg offered an almost unparalleled opportunity to study sporangia seemingly unaffected by host reaction. Eggs were inoculated in the chorioallantoic membrane with chlamydospores obtained from in vitro cultures. In a series of 177 fertile chicken eggs inoculated with *C. immitis*, between the fifth and 16th day of incubation, the fungus was isolated or identified in tissues in 81.5% of the inoculated eggs. Massive numbers of organisms in the sporangial stage were found in the allantoic fluids and in smaller numbers in tissue sections, extraembryonic membrane, and in mounts of yolk sac and albumen. However, it was noted that the mycelial stage frequently could be found on the membrane of the air sac. It was her impression that the chick embryo was an inert host. Several differences were observed between the sporangia cultured in fertile chicken eggs and those found in experimental animals. Spherules with endospore formation were found occurring up to 240 μ in diameter. Odd shapes were described with very thin walls and pseudopod-like structures, heavy crosswalls, absence of large central vacuole, and a considerable variability in staining reactions with Mallory's triple stain. She suggested that with the use of embryonated eggs a technique could be developed for the evaluation of various drugs for their effectiveness against the animal phase of *C. immitis* and that there was a possibility of collecting large numbers of the parasitic phase of the organisms for immunological, serological and physiological studies.

Recently, Brueck and Buddingh[3] reported their experiences with the propagation of pathogenic fungi in the yolk sac of embryonated eggs. A rapid and almost complete reversal into the spherule phase was noted to occur when suspensions of the mycelial growth of cultures of *C. immitis* were inoculated into the embryonic yolk sac. They concluded that the yolk sac of the developing chick embryo has proved to be highly suitable for the propagation of *C. immitis*.

Because of the potentialities inherent in the use of embryonated eggs for culturing *C. immitis* in the spherule stage, it was our feeling that this method warranted further investigation.

METHODS

1. Eight-day-old embryonated eggs were used throughout these experiments.

2. A 2 mm opening was made through the egg shell over the allantoic cavity or the yolk sac, depending upon the desired site of inoculation. Openings were made with a small electric hand drill with a burr of appropriate size.

3. Three sources of inoculum were used: (A) A saline suspension of pus obtained from a patient with coccidioidomycosis. (B) Saline suspensions of mouse omentum. These mice had been inoculated intraperitoneally with a saline suspension of arthrospores of *C. immitis*. After a two-week period the mice were sacrificed, the omentums removed aseptically, and a saline suspension was made of this material. (C) A suspension of arthrospores was made by overlaying with saline a test tube culture of mature *C. immitis* grown on Sabouraud's medium. The tube was gently agitated for several minutes and the saline suspension was removed aseptically.

4. After inoculation the openings in the eggs were sealed with nail polish.

5. All eggs were incubated at 99.5 F and at a humidity which gave a reading on the wet bulb thermometer of 86 to 88 F.

6. Eggs were opened on the eighth day of incubation and material taken for examination and cultural studies.

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3. Brueck, J. W. and Buddingh, G. J. 1951, Propagation of pathogenic fungi in the yolk sac of embryonated eggs. Proc. Soc. Exper Biol. & Med. **76**(2): 258–261.

EXPERIMENTAL DATA

Procedure no. 1. The evaluation of the rate of growth of C. immitis *in embryonated eggs over an eight-day period*

Saline suspensions of arthrospores were used. The number of arthrospores per cubic centimeter of inoculum was derived by the usual serial dilution plating technique. Sabouraud's dextrose agar was used as a culture medium. One tenth cc of inoculum was injected into the yolk sac of each egg. After incubation the eggs were opened aseptically and a note was made of the viability of the embryos. A 0.5 cc sample was taken from each egg for culture on Sabouraud's medium. Included in each sample was material taken from the yolk sac, allantoic and amniotic cavities. A 1.0 cc syringe with an 18-gauge needle was used to obtain the samples. The remaining entire contents of the eggs were pooled and mixed thoroughly in a Waring blendor. Samples of this material were then taken for plating to determine the degree of multiplication. Eight such experiments were done and a total of 51 eggs were used.

TABLE 1.—*Increase in the number of* C. immitis *organisms over an eight-day period in embryonated eggs.*

Eggs injected	Live eggs	Positive live eggs	Dead eggs	Positive dead eggs	Organisms per cc of inoculum	Organisms inoculated per cc of eggs	Organisms per cc of harvest	Amount of multiplication
8	6	3	2	0	670	2.2	225	102
7	4	4	3	2	560	1.9	6000	3158
7	6	2	1	1	500	1.7	320	188
5	3	3	2	2	1240	4.1	16000	3902
6	4	2	2	0	132	0.4	5700	1425
6	4	1	2	1	1440	4.8	610	127
6	3	0	3	1	97	0.3	1190	3966
6	3	2	3	1	2900	9.7	40	4.12
		Total						
51	33	17	18	8				
		Percentages						
	64.7	51.5	35.3	44.4				

Each egg was inoculated with 0.1 cc of an arthrospore suspension. Each egg was considered to have a 30 cc usable average volume.

Table 1 summarizes the results of these experiments. The number of colonies counted per cc by the plating technique varied from 97 to 2900 in the inoculum. Using the same plating technique on the pooled eggs revealed an increase of from 4 to 4000 times the original inoculum. Sixty-five percent of the embryonated eggs survived the mechanical trauma of inoculation and the effects of the presence of *C. immitis* over the eight-day period. No correlation could be found between the survivability of the embryos and the number of organisms which were used in our inoculum. Roughly, 50% of the eggs inoculated were found to be positive upon individual subculture when 0.5 cc of egg material was used. This percentage was about the same regardless of whether the embryo was alive or dead at the time of the opening.

One must interpret these results with the realization that at best the use of the plating technique gives only a rough estimate of the number of arthrospores inoculated and the number of organisms recovered. In the preparation of arthrospore saline suspensions it was frequently noted that such suspensions contained grossly visible aggregations of arthrospores and hyphal elements. These generally plated out as individual colonies. It should also be noted that small 0.5 cc samples taken from individual eggs for culture on Sabouraud's medium did not necessarily reflect the over-all growth distribution of the organism in the remaining portion of the embryonated egg. In spite of these

sources of error it is apparent that the embryonated egg does not provide a medium for the luxuriant growth of *C. immitis*.

Procedure no. 2. Evaluation of the rate of growth of C. immitis *in nonembryonated eggs as compared with the rate of growth in embryonated eggs*

The same technique was used as in procedure no. 1 except for the source of inoculum, which was a saline suspension of mouse omentum. Three experiments were done, using a total of 18 embryonated and 18 nonembryonated eggs.

Table 2 summarizes the rate of multiplication of *C. immitis* in embryonated eggs as compared to its rate of growth in nonembryonated eggs under the same conditions. *C. immitis* developed at a more rapid rate in the nonembryonated eggs. There were from eight to 13 times as many colonies per cc of harvest in the nonembryonated egg when compared to the yield obtained from the embryonated egg. One must again interpret these results in the light of the limitations of the plating and cultural technique used. It was concluded that the embryonated egg possessed a mild inhibitory effect on the growth of *C. immitis*.

TABLE 2.—*Comparison of the rate of growth of* C. immitis *in nonembryonated eggs and embryonated eggs.*

Enbronated eggs injected	Non-embryonated eggs injected	Organisms per cc of inoculum	Positive embryonated eggs	Positive non-embryonated eggs	Organisms per cc of embryonated eggs at harvest	Organisms per cc of nonembryonated eggs at harvest
6	6	320,000	4	0	1600	17,600
6	6	130,000	4	4	4300	58,800
6	6	130,000	0	4	4800	40,000

Mouse omentum was used as the source of inoculum. Dose of inoculum was 0.1 cc per egg.

Procedure no. 3. The survivability of C. immitis *upon passage through a series of embryonated eggs*

The original source of inoculum was mouse omentum. Specimens were taken from the individual egg for culture on Sabouraud's medium and survivability of the embryos was noted. The contents of only the viable eggs were pooled and mixed in a Waring blendor. This material was then used for the next succeeding inoculation. An amount of 0.2 cc inoculum was used for both the original and succeeding inoculations. Inoculations were made into the yolk sac. Transfers were made at weekly intervals and eight such transfers were done, using a total of 56 eggs. A plate count was made of the original inoculum and the pooled contents of the viable eggs. The pathogenicity of the organism was evaluated in mice at the end of this series of transfers.

Table 3 summarizes the results of this serial passage of *C. immitis*. The organisms can be transferred from egg to egg, and there is a gradual increase in number up to the eighth passage as adaptation to environment is made. Overwhelming numbers of organisms were never encountered. The series was stopped after the ninth egg passage as

TABLE 3.—*Multiplication of* C. immitis *in embryonated eggs on serial passage.*

Eggs injected	Live eggs	Positive live eggs	Dead eggs	Positive dead eggs	Organisms per cc of inoculum	Organisms per cc of yield
6	2	1	4	4	186,000	7,500
9	7	4	2	1	7,500	9,800
6	5	1	1	0	9,800	18,000
6	4	1	2	1	18,000	26,000
6	5	4	1	1	26,000	37,000
5	3	2	2	1	37,000	42,800
6	4	2	2	1	42,800	44,800
6	4	1	2	1	44,800	2,500
6	2	0	4	0	2,500	2,300
			Total			
56	36	16	20	10		

Original source of inoculum was mouse omentum. The contents of only the viable eggs were pooled and counted. Two-tenths of inoculum was used for both the original and succeeding inoculations.

it was felt that no additional information could be gained. *C. immitis* was cultured in embryonated eggs for a total of 63 days. Egg material taken at this time and containing *C. immitis* was inoculated intraperitoneally into 12 mice. At the end of a two-week period, four of the mice had died and the remainder were killed. On autopsy all the mice were found to contain gross and microscopic evidence of coccidioidomycosis. It was concluded that at the end of the serial passage this organism had lost none of its virulence and proved to be as pathogenic to mice as those organisms in the original source of inoculum.

Microscopic examination of eggs inoculated with C. immitis *and control eggs*

Wet mount preparations were made from the yolk sac, chorioallantoic fluid, and albumen. These mounts were made from the eggs inoculated into the allantoic cavity and yolk sac from the three sources described under *Methods*. Material was examined from both viable and dead eggs. Microscopic examination using both the conventional and phase microscopes revealed numerous spherules. In size they varied from small, immature spherules to several times larger than any size reported for mature *C. immitis* spherules. Many of these spherules contained smaller spherules which caused them to closely resemble the sporangial state of *C. immitis* with endospore formation. Finally, spherules could be found in which the membranes had ruptured and the contents were being released in a manner similar to that seen in *C. immitis*. All of these spherules had relatively thin walls and were not infrequently irregular in shape. The largest number of spherules was found in the yolk sac and to a lesser extent throughout the remainder of the egg structure. In those eggs in which the embryo was found dead on opening, the spherules were more greatly dispersed throughout the egg. When wet mount preparations were made from noninoculated control eggs, these same spherules could be found in comparable amounts. Also, when pus containing known *C. immitis* spherules was mixed with these substances, in many instances it was impossible to determine whether the spherule under observation was one having its origin in pus or egg substance. Because of these observations, several staining methods were used in an effort to distinguish these spherules from the spherules of the animal phase of *C. immitis*.

The following stains were used: periodic-acid-Schiff stain,[4] Mallory's triple stain,[5] and hematoxylin and eosin stain. Smears were made from noninoculated eggs, eggs inoculated with *C. immitis* and pus containing *C. immitis* spherules, the latter mixed with various portions of noninoculated eggs and stained for comparison. With the periodic-acid-Schiff stain, the spherules in pus stained red or shades thereof while the remainder of the pus constituents stained blue. The spherules revealed ridging of their surface due to rapid dehydration occurring during the preparation of the smear. Most of the stain appeared to be absorbed by the wall of the spherule, and it was difficult to identify the contents of the spherule. Smears from control eggs treated with this stain revealed many spherules with the same staining characteristics and shape as those in pus. Identification was impossible on

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5. McClung's Handbook of Microscopial Technique. 1950, ed. 3, Paul B. Hoeber, Inc.

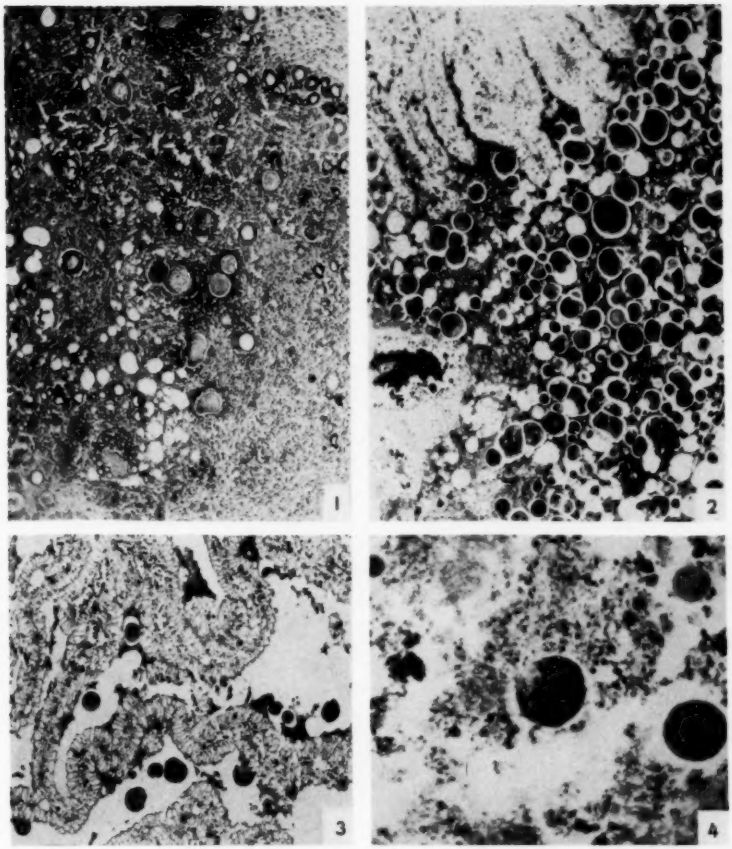

FIG. 1.—Hematoxylin and eosin stained preparation of omentum from a mouse injected intraperitoneally with pus containing *C. immitis* spherules. Mice sacrificed two weeks after inoculation. Specimen magnified 70 times.

FIG. 2.—Hematoxylin and eosin preparation of yolk from control embryonated chicken egg showing numerous spherules resembling the animal phase of *C. immitis*. Specimen magnified 70 times.

FIG. 3.—Hematoxylin and eosin preparation of the extra-embryonic membranes from an uninoculated embryonated chicken egg showing spherules. Specimen magnified 70 times.

FIG. 4.—Spherules from uninoculated embryonated chicken eggs resembling endosporulation. Specimen magnified 350 times.

color characteristics alone in those smears where noninoculated egg material and pus were combined. It was impossible to positively identify *C. immitis* spherules which may have been present in the inoculated eggs by the use of this stain.

Spherules in pus stained red to orange, and the remainder of the pus constituents stained blue with the use of Mallory's triple stain. Smears of control eggs stained by this method revealed spherules which stained blue, red, and red-brown, with a bluish back-

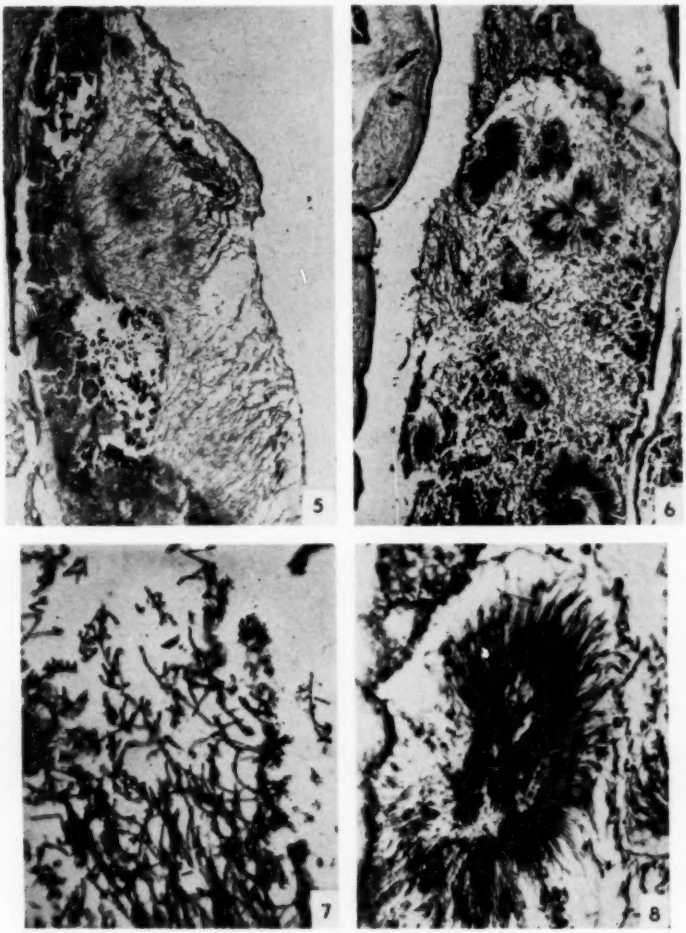

FIG. 5.—Hematoxylin and eosin preparation of extraembryonic membranes from inoculated egg showing nests of *C. immitis* in the mycelial stage. This material was taken from an inoculated egg which was dead on opening. Specimen magnified 70 times.

FIG. 6.—Hematoxylin and eosin preparation of extraembryonic membranes from inoculated egg showing nests of *C. immitis* in the mycelial stage associated with asteroid bodies. This material was taken from inoculated embryo which was viable on opening. Specimen magnified 70 times.

FIG. 7.—Enlargement of mycelial matt of *C. immitis*, showing individual hyphal elements with no tendencies toward spherule formation. Specimen magnified 350 times.

FIG. 8.—Asteroid-like body associated with mycelial elements. These were usually found in the allantoic cavity. Specimen magnified 350 times.

ground. When pus containing *C. immitis* spherules was mixed with noninoculated egg material and stained, it was impossible to identify most individual spherules. It was also impossible to positively identify *C. immitis* spherules

which may have been present in the inoculated eggs with the use of this stain.

Essentially the same difficulty was encountered with the use of the hematoxylin and eosin stain.

Small samples of yolk, albumen, extraembryonic membrane and various portions of the embryo per se were placed in a 10% formaldehyde solution for histological sectioning and staining. Microscopic examination of stained histological sections of various portions of uninoculated eggs revealed numerous spherules similar to those noted in wet mount and stain smear preparations (see fig. 3 and 4). The yolk substance was composed almost entirely of these spherules. However, they could be found in lesser numbers in sections made of extraembryonic membrane, albumen, and in various portions of embryos found dead upon opening.

Figure 1 is a photograph of a section of omentum from a mouse previously inoculated with pus containing *C. immitis* and is presented for comparison. It was photographed at the same magnification as figures 2 and 3.

Eggs which were inoculated with *C. immitis* from the three sources mentioned under *Methods* were found upon opening to contain thickened, chorioallantoic membranes and diffuse, white, matt-like material dispersed between the extraembryonic membranes and embryo. This material was slightly adherent to the surrounding structures. These observations were most frequently noted in those embryos which were viable at the time of opening. Microscopic examination revealed this material to consist of closely woven matts of hyphal elements of *C. immitis*. No tendency to spherule formation could be observed (fig. 5, 6, and 7). Large eosinophilic star-shaped bodies were seen associated with the mycelial elements in many instances (fig. 6 and 8.) These bodies were composed of a basophilic center which was surrounded by radiating eosinophilic noncellular strands of material, and the whole structure was slightly suggestive of the asteroid bodies which have been noted with various types of the deep mycotic infections of man.

Microscopically, loosely intertwining matts of mycelial elements were found in both viable and dead embryos at the time of opening. Mycelial elements were found regardless of the source of inoculum. Microscopic examination of tissue sections of embryos per se which were viable at the time of opening revealed no evidence of spherules or mycelial elements. *C. immitis* in these eggs was present in the yolk sac, albumen, and extraembryonic membranes.

SUMMARY

Several studies have recently appeared in the literature suggesting the embryonated chicken egg as an excellent laboratory animal for the cultivation of large numbers of animal phase of *Coccidioides immitis*. Our investigations have failed to substantiate these observations. Plate counts of the inoculum and yield did not reveal a tremendous growth of *C. immitis*. When plate count harvests were compared between embryonated and nonembryonated eggs, it was found that embryonated eggs possessed a slight inhibitory effect on the rate of growth of *C. immitis*. *C. immitis* was transferred through nine egg passages for a total of 63 days. During the early transfer the rate of growth increased but did not reach a degree which would indicate that the organism was in a favorable environment. *C. immitis* organisms which had been cultured in embryonated eggs for a period of 63 days were found to have maintained their same degree of pathogenicity when injected into mice.

Microscopic examination of various portions of control eggs revealed numerous spherules. The yolk is composed almost entirely of these spherules, which represent aggregates of nutrient substances. They resemble the spherules of *C. immitis* in that they are spherical, of similar size, possess a membrane, contain granules, and stain similarly. They differ from the spherules of *C. immitis* in that many of the globules are of a much larger size than previously observed for *C. immitis*; their membranes are thin; bizarre forms are noted; and a variable staining pattern is observed. Those stains used were of no differential aid.

It is our belief that under certain conditions and for short periods of time *C. immitis* when inoculated into embryonated eggs may tend towards spherule formation. However, we believe that this is exceptional. Regardless of the source of inoculum, whether mouse omentum, human pus, or the use of a suspension of arthospores, *C. immitis* was found to be growing in the mycelial form. In those embryos found viable at the time of opening, the embryo was not found to be infected. In these eggs *C. immitis* existed in the yolk sac, allantoic cavity, and other extraembryonic structures as loose matts composed of hyphal elements. Similar findings were seen in those embryos found dead on opening.

SUPERINFECTION IN EXPERIMENTAL BRUCELLOSIS OF GUINEA PIGS

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It has long been accepted that in tuberculosis and syphilis there is resistance to reinfection. Factors of reinfection in brucellosis, a disease resembling tuberculosis and syphilis, are not well defined. Römer[11] showed that virulent tubercle bacilli of bovine type inoculated into guinea pigs with tuberculosis produced by attenuated organisms of human type, did not cause miliary tuberculosis as seen in controls, but produced a modified form of the primary infection. After delayed death, the second infecting organism was recovered only from the site of injection, whereas the first infecting organism was recovered from chronic fibrocaseous pulmonary lesions. The initial tuberculous infection conferred resistance against subsequently inoculated tubercle bacilli but not against other bacteria.

In experimental syphilis,[7] syphilitic rabbits developed a second chancre when inoculated with a heterologous but not with a homologous strain of *Treponema pallidum*. It was also found by subinoculation experiments that the occasional syphilitic rabbit which failed to develop a chancre following superinfection with heterologous treponemata could harbor both infecting organisms within the regional lymph nodes. It was concluded that existing syphilitic infection did not hinder the invasion of superimposed treponemata, but did protect against the development of new lesions.

With respect to brucellosis, several investigations have shown that artificial infection with virulent[1,5] or avirulent *Brucella abortus*[3] followed by reinfection with virulent brucellae reduced the incidence of abortion in cattle. This has been accepted as evidence for resistance. Large scale immunization of cattle with living brucella vaccine is now widely practiced.

On the basis of bacteriological culture of tissues from guinea pigs reinfected with brucellae, several authors[4,6,9] have

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3. Cotton, W. E., Buck, J. M. and Smith, H. E. 1933, Efficacy and safety of abortion vaccines prepared from *Brucella abortus* strains of different degrees of virulence. J. Agric. Res. **46**: 291–314.
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8. McCullough, W. G., Mills, R. C., Herbst, E. J., Roessler, W. G. and Brewer, C. R. 1947, Studies on the nutritional requirements of *Brucella suis*. J. Bact. **53**: 5–15.

reported increased resistance in animals primarily infected with strains of low or high virulence. McEwen,[9] infecting guinea pigs with a carbon dioxide-dependent strain and reinfecting with an aerobic strain of *Br. abortus*, found evidence of resistance one to 26 weeks after the primary infection.

Similar experiments were undertaken to determine whether guinea pigs infected with virulent brucellae were resistant to superinfection with another species of virulent brucellae. Infection with one or the other organism could be detected not only by their cultural requirements but also by their different pathological effects.

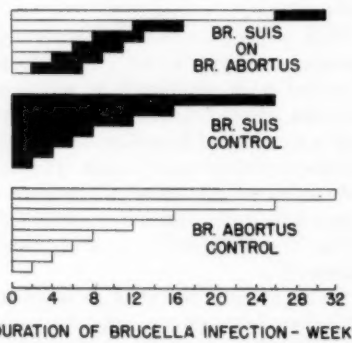

FIG. 1.—Brucella superinfection. Plan of experiment.

MATERIALS AND METHODS

Animals.—Guinea pigs of both sexes were of the Hartley strain and weighed 300 to 425 g.

Bacteria.—*Br. abortus* 6232c sr3 was a streptomycin-resistant mutant isolated from a smooth strain. The ID_{50} of this organism for guinea pigs was 1160 organisms. *Brucella suis* PSIII k was a smooth virulent strain. The ID_{50} of this organism for guinea pigs was three bacteria. Stock cultures were maintained on modified tryptose agar.[8] Bacterial suspensions for injection into animals were made from 48-hour tryptose agar slant cultures suspended in tryptose saline consisting of 0.1% tryptose in 0.5% sodium chloride. The suspensions were standardized in the Coleman spectrophotometer and checked by the dilution plate count method.

Autopsies.—Animals were bled from the heart under chloroform anesthesia and then electrocuted. Autopsies were performed under aseptic conditions. A complete morphological examination was made on almost all animals. Tissues were fixed in Helly's solution and in formalin. Sections were stained by a modified Lillie-Giemsa method.

The regional lymph nodes and spleen were cultured in all animals. In many cases other lymph nodes, liver, uterus, or epididymis were also cultured. Tissues to be cultured were triturated aseptically with tryptose saline, and two or three drops of the resulting suspension were spread on agar plates. Since the strain of *Br. abortus* used was resistant to streptomycin and required carbon dioxide for growth, it could be readily differentiated from the streptomycin-sensitive *Br. suis* growing without added carbon dioxide. Cultures from animals infected with both species were plated in duplicate on 2-1 agar[2] and on 2-1 streptomycin agar (1 mg streptomycin per ml). One plate of each type of agar was incubated aerobically, the other in a candle jar. Only *Br. abortus* would grow on the streptomycin-containing agar incubated in jars and only *Br. suis* on the 2-1 agar cultivated in air. To detect possible changes in cultural characteristics during host passage, 2-1 agar in carbon dioxide and streptomycin agar in air were employed. Cultural changes were not found. Except for occasional check plates, tissues from control animals infected with a single species were cultivated only under the conditions appropriate for the strain used. All cultures were examined after four days incubation at 37 C.

Agglutinin tests were performed with blood serum obtained from each animal when sacrificed. The antigen employed was a phenolized saline suspension of *Br. abortus* produced by the U. S. Army Veterinary School.

EXPERIMENTAL

The plan of the experiment is represented in figure 1.

Infection controls.—*Br. abortus* was injected subcutaneously in the right groins of 60 guinea pigs in a dose of 6900 organisms in 0.2 ml tryptose saline. Groups of five animals were sacrificed at intervals of two, four, six, and eight weeks and groups containing ten ani-

9. McEwen, A. D. 1940, Immunity to *Brucella abortus* infection. Vet. Rec. **52**: 441–444.

mals at 12, 16, 26, and 32 weeks after infection.

Br. suis was injected subcutaneously in the right groins of 50 guinea pigs in a dose of 34 organisms in 0.2 ml tryptose saline. The planned dose was 30 organisms. The animals were sacrificed at the same intervals as those infected with *Br. abortus*, except that none was examined at 32 weeks.

Superinfection.—Sixty guinea pigs received the same dose of the same suspension of *Br. abortus* as the *Br. abortus* controls. Groups of ten were injected subcutaneously with *Br. suis* at intervals of two, four, six, eight, 12 and 26 weeks after infection with *Br. abortus* and sacrificed five weeks after reinoculation. To distinguish between the local tissue reactions caused by each of the organisms, *Br. suis* was injected into the left axilla, a site far removed from the previous *Br. abortus* injection. Separate tests of the *Br. suis* inoculums at the eight-, 12- and 26-week periods produced infection in 26 of 27 normal guinea pigs.

RESULTS

The gross morphological findings in animals examined from two to 32 weeks after infection were distinctive in the regional lymph nodes and spleen. Figures 2 and 3 summarize graphically the relative size of lymph nodes and spleen in the three types of infection at various periods from two to 32 weeks. The results are plotted according to the total duration of brucella infection. The age of the *Br. abortus* infection at the time of superinfection is obtained by subtracting five weeks, the period of superinfection. The figures are drawn only from those animals which had complete morphological examinations and indicate cultural results which are referable specifically to regional lymph nodes and spleen and not to the whole animal.

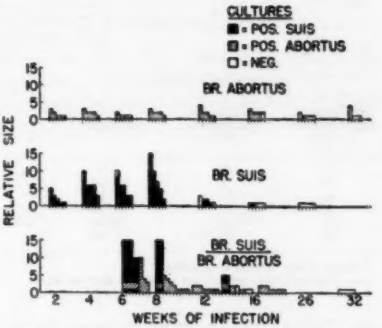

FIG. 2.—Regional lymph node size during infection with *Br. abortus*, *Br. suis*, or *Br. suis* superimposed on *Br. abortus*. Number of animals is indicated by number of segments on ordinates.

The bacteriological results from all experimental animals appear in tables 1 and 2. The incidence of positive cultures is greater in the tables than in figures 2 and 3 because more animals are represented in the tables. In addition, positive cultures were occasionally obtained from tissues other than regional lymph nodes and spleen when the latter were negative.

Br. abortus *control infection.*—There was only slight enlargement of regional

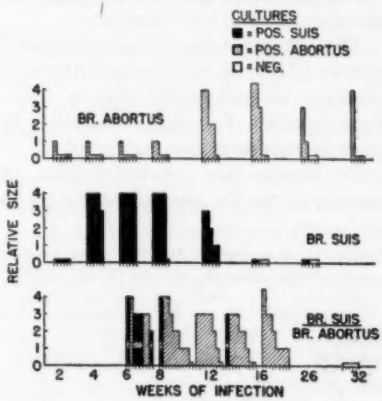

FIG. 3.—Spleen size during infection with *Br. abortus*, *Br. suis*, or *Br. suis* superimposed on *Br. abortus*. Number of animals is indicated by number of segments on ordinates.

TABLE 1.—*Recovery of brucellae from guinea pigs infected with* Br. abortus *and superinfected with* Br. suis.

Br. abortus infection*			*Br. suis* infection			
Duration at superinfection (weeks)	Duration at sacrifice (weeks)	Incidence	Dosage, *Br. suis*	Duration at sacrifice (weeks)	Incidence	
					Superinfected animals	Inoculum controls†
2	7	9/9	28	5	5/9	
4	9	10/10	27	5	2/10	
6	11	10/10	38	5	0/10	
8	13	10/10	29	5	2/10	10/10
12	17	10/10	21	5	0/10	10/10
26	31	2/10	17	5	0/10	6/7

* Dose of *Br. abortus* was 6900.
† Included to test infectivity of superinfecting dose.

and distant lymph nodes through the period of observation. The spleens, small during the first eight weeks of infection, became very large at 12 weeks and extremely large at 16 weeks. Most of them returned to normal size at 32 weeks. Specific lesions were not observed grossly in other viscera before 26 weeks. Liver abscesses were seen in two animals at 26 weeks. One animal at 32 weeks showed metritis as well as liver abscess.

Histological examination revealed the granuloma of brucellosis in regional and distant lymph nodes, spleen, liver and bone marrow. These lesions were moderate in number and, unlike those in *Br. suis* infection, lacked necrosis.

The extreme enlargement of spleens seen at 12 and 16 weeks was of the type observed in nonspecific chronic infectious splenitis. Specific granulomata in these spleens were few and small.

Br. abortus was recovered from all animals in the *Br. abortus* control group through 12 weeks of infection. The incidence of recovery diminished thereafter. By 26 weeks the organism could be recovered from only half the animals.

TABLE 2.—*Recovery of brucellae from controls infected with either* Br. abortus* *or* Br. suis.*

Br. abortus		*Br. suis*	
Duration at sacrifice (weeks)	Incidence	Duration at sacrifice (weeks)	Incidence
2	5/5	2	5/5
4	5/5	4	5/5
6	5/5	6	5/5
8	5/5	8	5/5
12	10/10	12	10/10
16	8/10	16	6/10
26	5/10	26	6/9
32	5/10	32	

*Dose of *Br. abortus* was 6900, of *Br. suis* 34.

Br. suis *control infection.*—Maximal lesions developed in from four to eight weeks. Not only were the regional lymph nodes and spleens greatly enlarged but they had large necrotic granulomata. Similar lesions were found microscopically in epicardium, lung, liver, renal pelvis, renal cortex, mesovarium, uterus, testis, epididymis, thyroid, adrenal, bone marrow, bone, and intestine. At 16 to 26 weeks, lymph nodes and spleens had returned to normal size but contained grossly visible abscesses and, microscopically, fine scarring and fibrosis.

At four to eight weeks, the regional lymph nodes and spleens of *Br. suis* infection were enlarged with confluent necrotic granulomata, while those of *Br. abortus* infection were scarcely altered. These gross differences clearly distinguished the two types of infection. Because of these morphological differences, known from previous experience, five weeks had been selected as the interval between *Br. suis* inoculation and sacrifice of all animals in the superinfection experiments.

The cultural recovery of *Br. suis* from the *Br. suis* control group was similar to that of *Br. abortus* just described. In the infections of longer duration organisms were found in a greater number

of animals by culturing not only regional lymph nodes, spleen and liver but also any other areas with gross inflammation. For example, nine animals autopsied and cultured after 26 weeks of *Br. suis* infection revealed the following: Six had positive *Br. suis* cultures. Of five with negative cultures from the regional lymph nodes, two had positive cultures from the spleen. One with negative cultures in regional lymph nodes and spleen had positive cultures in cervical lymph nodes and epididymis. In infections of more than four months duration, histological evidence of active brucellosis could be found when cultures of tissues were negative.

Br. suis *superimposed on* Br. abortus *infection.*—When *Br. suis* was superimposed on two-weeks-old *Br. abortus* infection, great enlargement of regional lymph nodes and spleens ensued in all animals. More significantly, the spleens had the large, confluent granulomata characteristic of *Br. suis* infection. Disseminated visceral lesions were fewer and less intense than in control *Br. suis* infection, and *Br. suis* could be cultured from only five of the nine animals.

When *B. suis* was superimposed on *Br. abortus* infection varying in duration from four weeks to 26 weeks, morphological evidence of superinfection was found in only four of 50 guinea pigs. These occurred in groups inoculated with *Br. suis* four weeks and eight weeks after *Br. abortus* infection. All four superinfections were recognized grossly by changes characteristic of *Br. suis* infection. Positive *Br. suis* cultures were obtained from the regional lymph nodes of all four guinea pigs but from only two of the spleens. Animals resistant to *Br. suis* infection six to 12 weeks after *Br. abortus* infection had large spleens similar to those of the control *Br. abortus* infections. In the superinfection groups. discrepancies again appeared between the results of morphological and of bacteriological examinations. More evidence for *Br. suis* infection appeared from the anatomical than from the cultural results. Although only five of nine guinea pigs superinfected with *Br. suis*

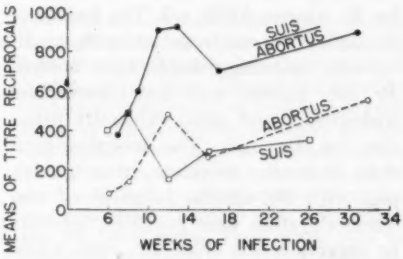

FIG. 4.—Mean serum brucella agglutinin titers in guinea pigs infected with *Br. suis*, *Br. abortus*, or *Br. suis* superimposed on *Br. abortus*.

after two weeks of *Br. abortus* infection yielded positive cultures, all had morphological evidence of *Br. suis* infection.

It is noteworthy that *Br. abortus* was isolated from all animals in each of the superinfection groups save those examined at 31 weeks, at which time *Br. abortus* was recovered from two of ten animals and *Br. suis* from none. However, at this time a culturally negative animal examined histologically showed evidence of active *Br. abortus* infection.

Serum agglutinins.—Serum agglutinin titers for brucella were determined in each animal at the time of sacrifice. The arithmetic means of the reciprocals of the titers in each group at the various intervals after infection are plotted in figure 4. The mean serum agglutinin levels of guinea pigs in which *Br. suis* was superimposed on *Br. abortus* infection were significantly higher than those of animals infected with either *Br. abortus* or *Br. suis*. Agglutinin titers rose after reinoculation but were not correlated with morphological or bacteriological evidence of superinfection. Animals showing superinfection had titers

which were no different from those of animals which were refractory.

DISCUSSION

It is apparent that morphological differences existed between cavine brucellosis caused by *Br. suis* PSIII k and by *Br. abortus* 6232c sr3. The lesions of *Br. suis* in superinfected animals, qualitatively indistinguishable from control *Br. suis* lesions, were less intense, less widespread, and often culturally negative for *Br. suis*. The morphological data indicate, therefore, that guinea pigs with *Br. abortus* infection of two weeks duration were partially resistant to superinfection with *Br. suis*, while guinea pigs with *Br. abortus* infection of longer duration were refractory with few exceptions.

Although *Br. abortus* was recovered for 17 weeks from all animals in which superinfection was attempted, it was recovered from only two of ten animals 31 weeks after primary infection. This suggests that resistance was not dependent on survival of organisms causing the original infection. On the other hand, failure to recover *Br. abortus* in late infections might reflect the inadequacy of cultural methods in detecting small numbers of organisms in tissues. It is possible that in all experiments of this type subinoculation or better cultural technique might reveal persisting organisms. Whether the *Br. suis*-resistant animals had indeed rid themselves of *Br. abortus* must remain an unanswered question.

By culturing only spleens, McEwen[9] concluded that guinea pigs infected with large doses of one virulent strain of *Br. abortus* were resistant to superinfection with large doses of another strain of the same species. However, in later experiments,[10] culture of the regional lymph nodes revealed the second organism. The present studies extended the observations of McEwen in showing that resistance to superinfection also occurred when different species of brucella were used for the first and second infections and when the primary infecting dose was relatively small.

SUMMARY AND CONCLUSIONS

1. *Brucella suis* PSIII k and a streptomycin-resistant strain of *Brucella abortus*, 6232c sr3, both virulent organisms, differed in their pathological effects in guinea pigs.

2. Guinea pigs having brucellosis due to *Br. abortus* were inoculated with *Br. suis* at various intervals up to six months following the original infection. When present, superinfection could be detected by the morphological changes in the host and by the cultural characteristics of the recovered organisms.

3. On the basis of pathological evidence, guinea pigs with *Br. abortus* infection of two weeks duration were susceptible to infection with *Br. suis*. However, only one-half of them yielded *Br. suis* when cultured. Thereafter, *Br. abortus*-infected guinea pigs were largely refractory to *Br. suis* infection.

4. During the first 17 weeks of brucellosis, *Br. abortus* was recovered from all animals resistant to *Br. suis* superinfection, while at 31 weeks *Br. abortus* was recovered from only 20% of resistant animals.

5. Whether or not superinfection occurred following inoculation of *Br. suis* into *Br. abortus*-infected animals, serum agglutinins for brucellae increased above the level found in control *Br. abortus* infections.

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INTERFERENCE OF AUREOMYCIN WITH THE ACTIVITY OF PENICILLIN IN VIVO

PASQUALE BARTELL

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Gunnison et al[1] have shown that aureomycin has an inhibitory effect on the activity of penicillin in vitro. This fact had previously been confirmed in this laboratory. The present data are derived from experiments designed to determine if this interference could be demonstrated in vivo and the factors which influence such interference.

MATERIALS AND METHODS

The test organism employed throughout this study was a 14- to 18-hour growth of *Diplococcus pneumoniae*, type I, cultured in brain-heart infusion broth (Difco) enriched with 5% defibrinated rabbit blood, pH 7.0. This strain was inhibited at concentrations of 0.03 units/ml penicillin and by 0.15 mg/ml aureomycin in vitro.

The antibiotics used were crystalline sodium penicillin G and aureomycin hydrochloride. These antibiotics were dissolved in distilled water and used fresh whenever possible. Otherwise they were stored at −20 C and used within a few days. When simultaneously administered, the antibiotics were injected intramuscularly into opposite thighs.

Healthy young white mice which weighed 15 to 20 g were employed. The mice were used in groups of five, and each assay was repeated at least three times. In general, the procedure[2] was as follows. Mice were infected by intraperitoneal inoculation with 0.5 ml of a 1:10 dilution of a 14- to 18-hour culture of *D. pneumoniae*, type I, containing 1.0×10^8 organisms, approximately one hour before administration of the antibiotics. The course of the infection was followed by determining the number of bacteria per drop of infected mouse blood (approximately 0.03 ml) at one, three, five, eight, and 24 hours after the administration of the antibiotics. This was done in the following manner: A drop of blood procured from the tail of the mouse was added to 2.0 ml of plain nutrient broth. This was well mixed by repeated drawing back and forth in a pipette. One ml of this mixture was withdrawn and tenfold dilutions were prepared in plain nutrient broth. Pour plates were made by adding 1.0 ml of the undiluted and 10^{-1} dilution to 15 ml blood agar base (Difco) enriched with 0.5% glucose and 2% horse serum. These plates were incubated for 48 hours at 37 C. Colony counts were made using the Quebec Colony Counter.

RESULTS

In figure 1 it can be seen that penicillin produced a decrease in the bacteremia more rapidly than did aureomycin. Since the bacterial count increased rapidly after the seventh hour, it is evident that the effective antibacterial blood level of penicillin was not maintained beyond this hour. Aureomycin, on the other hand, maintained an effective antibacterial activity as indicated by the constant decrease in the bacterial count. When these antibiotics were injected simultaneously an interference upon the activity of penicillin resulted. This interference was evident during the first five to seven hours.

It is evident then, that penicillin when administered simultaneously with aureomycin does not exhibit its characteristic activity. It appears, however, that this interference is of a temporary nature.

It is shown in figure 2 that when aureomycin was administered prior to the administration of penicillin the resultant curve resembled more closely the curve representing a single injection of aureomycin. When, on the other hand,

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penicillin was administered prior to aureomycin, the resultant curve resembled the curve representing a single injection of penicillin. In both cases, interference on the activity of penicillin was evident, but to different degrees.

This experiment makes it quite clear that the sequence of administration of the antibiotics, with some time interval between injections, does have a definite effect upon the degree of interference exhibited. When penicillin is administered prior to aureomycin, there is a diminution in the degree of interference. When aureomycin is administered before penicillin, there is an increased degree of interference.

When penicillin was administered prior to aureomycin there resulted a variance in the degree of the interference, depending upon the length of the time interval between injections. The degree of interference appeared to be greater with the shortening of the interval of time between the administration of penicillin followed by aureomycin. This is indicated in figure 3. Conversely, a lesser degree of interference was evident when penicillin was permitted to act alone for a longer interval of time before the administration of aureomycin.

In performing survival experiments, it can be seen in figure 4 that the simultaneous use of aureomycin and penicillin (C) resulted in an additive effect.

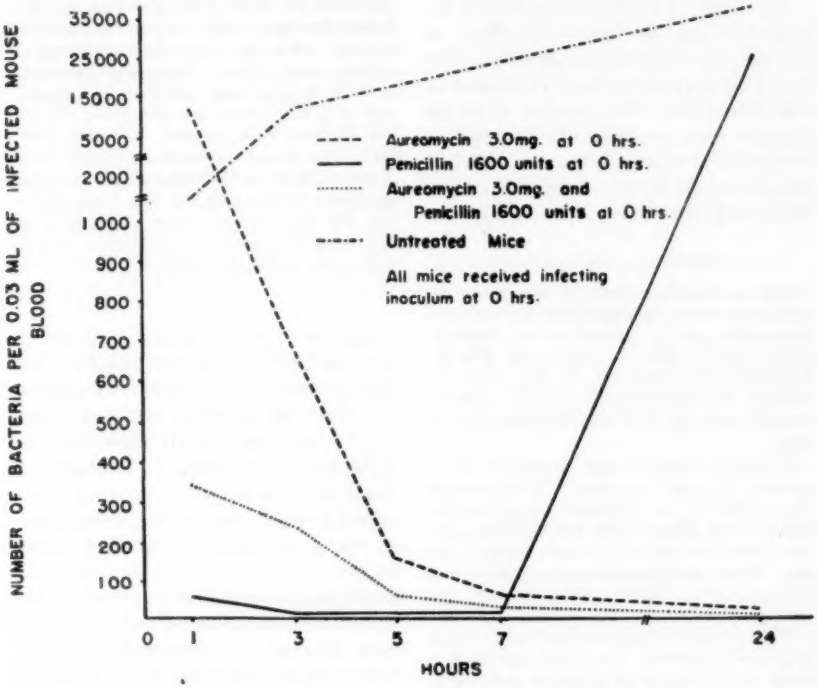

FIG. 1.—Survival of *D. pneumoniae*, type I, in mice after simultaneous administration of aureomycin and penicillin.

When aureomycin was administered four hours after the initial injection of penicillin (D) the mice had a survival rate which was twice as great as that obtained when using the same antibiotics simultaneously. The use of penicillin followed in four hours by a similar injection of penicillin (E) was not as efficacious as the single injection of aureomycin (A).

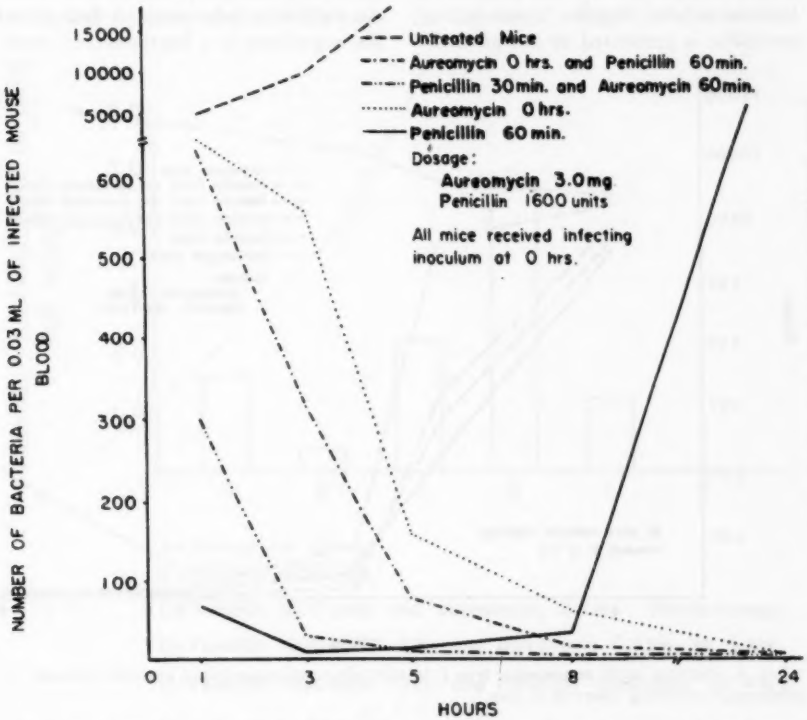

FIG. 2.—Survival of *D. pneumoniae*, type I, in mice after administration of aureomycin and penicillin in varying sequence.

The interference which was previously noted is not made evident in the survival experiment (fig. 4, A,B,C), unless the optimal conditions necessary for maximal efficiency of these antibiotics are realized. The optimal conditions entail the administration of penicillin four hours before the administration of aureomycin (fig. 4,D). It will be noted that under such conditions the mice showed a survival rate which was twice as great as that obtained by using the same antibiotics simultaneously. This would indicate that although an additive effect was obtained as a consequence of simultaneous administration of the antibiotics, the full potential of the combination was not attained.

DISCUSSION

When aureomycin is administered in combined therapy with penicillin there

occurs a temporary, but definite, interference upon the action of penicillin (fig. 1). As was shown, the degree of interference is altered by the sequence and the time of administration (fig. 2 and 3).

A study of the combined use of these antibiotics shows that the greater the interval of time that the "faster-acting" penicillin is permitted to act alone before the administration of aureomycin, the less is the degree of interference exhibited (fig. 2 and 3). On the other hand, when the "slower-acting" aureomycin is permitted to exert its influence for a period of time before the administration of penicillin, the greater is the degree of interference on the action of penicillin (fig. 2).

To explain how the antibacterial activity may be modified, it must be assumed that the antibiotic administered first does not act upon the entire bacterial population. With this assumption then, the second antibiotic engages only those bacteria which have not already come under the influence of the antibiotic administered first.

In order to facilitate interpretation of the data presented it might be said that the antibiotic administered first places the organisms in a bacteriostatic state. Once this occurs, the second antibiotic is ineffective against those organisms in a state of stasis. It has been shown that organisms must be actively multiplying for aureomycin to be effective,[3] and this has also been shown to be true in the case of penicillin.[4,5] Thus, the rate

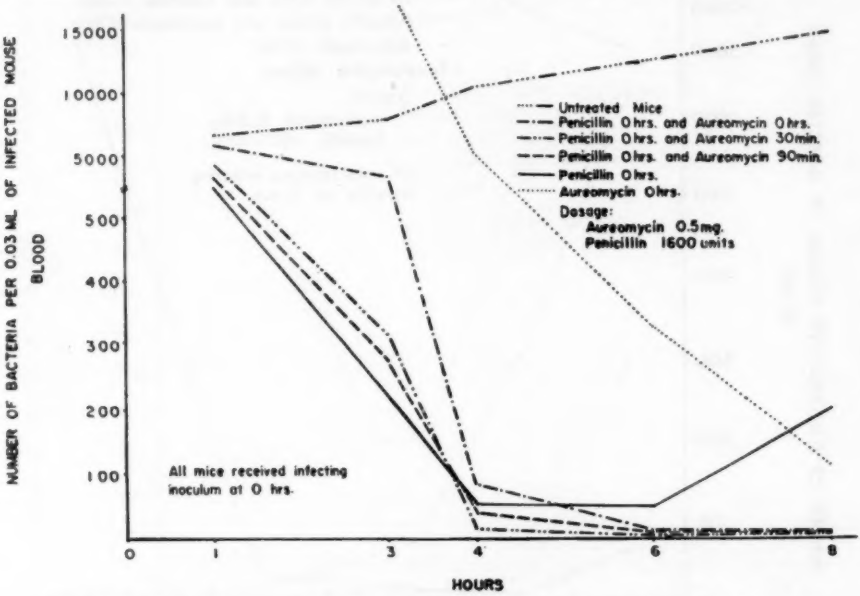

FIG. 3.—Survival of *D. pneumoniae*, type I, in mice after administration of penicillin followed by aureomycin at varying intervals of time.

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of combined activity may be dependent, in part, upon the type of antibiotic which is administered first, and the length of time it is permitted to act before administration of the second antibiotic.

The above interpretation may also be applied to those results which were obtained in the survival experiments. As can be seen in figure 4, the combined use of aureomycin and penicillin is at least additive. Here, the penicillin sharply reduces the bacterial count, and aureomycin, being present after the excretion of penicillin, further reduces the bacterial count, thus adding to the chance for survival. Although this result is favorable, it is not the method which achieves maximal efficiency. It is seen that an even greater number of survivals results when aureomycin is administered four hours after the administration of penicillin. The administration of the antibiotics in this manner avoids the interference which is otherwise indicated (fig. 1). This can be seen by a comparison of lines C and D of figure 4. This is further substantiated by an analysis of figure 3, where it will be noted that the longer penicillin acts alone before the administration of aureomycin, the more efficient is the antibacterial activity.

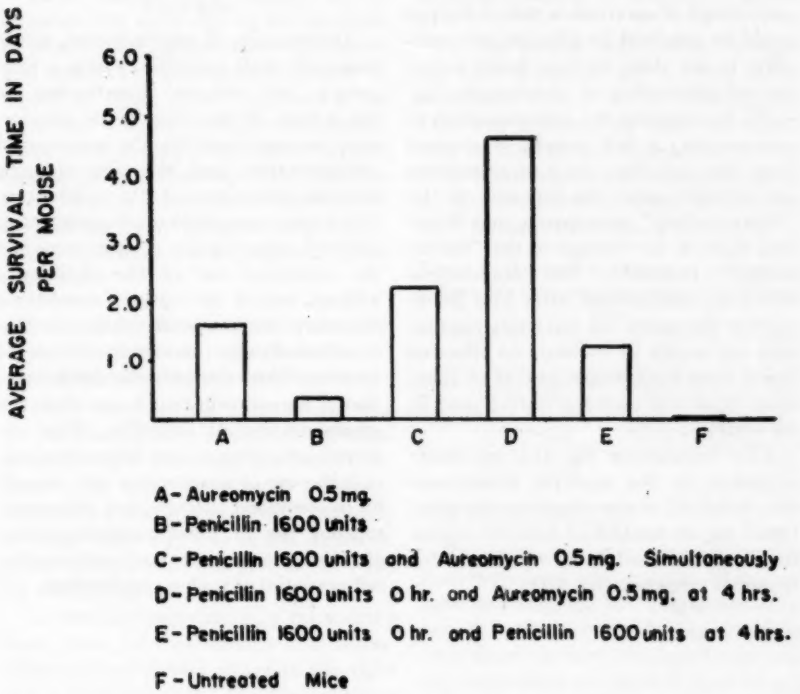

FIG. 4.—Average survival time of mice treated with penicillin and aureomycin, singly and combined, after injection with *D. pneumoniae*, type I.

To explain further the marked dif-

ference of survivals as represented by lines C and D of figure 4, it might be said that when the antibiotics are administered simultaneously, both penicillin and aureomycin act on some portion of the total bacterial population. However, during the period of interference (fig. 1) some of each antibiotic is being lost via excretion. Therefore, the percentage of survivals is not as high as could be obtained by allowing the penicillin to act alone for four hours before the administration of aureomycin (fig. 4,D). By delaying the administration of aureomycin, a full benefit is derived from the penicillin, since no organisms are brought under the influence of the "slower-acting" aureomycin, and therefore there is no wastage of the "faster-acting" penicillin. The aureomycin which is administered after four hours further decreases the bacterial population and is able to maintain an effective blood level for a longer period of time, since none was excreted during penicillin activity.

This interference (fig. 1) is not made apparent in the survival experiment (fig. 4,A,B,C) if one considers the combined use of antibiotics without regard for optimal conditions necessary for maximal efficiency (fig. 4,D).

The interference which has been observed in this work does not contraindicate the use of aureomycin and penicillin in combined therapy. However, by being aware of this interference it then becomes possible to realize the full potential of these antibiotics when they are used in combined therapy.

SUMMARY

Aureomycin, if administered simultaneously with penicillin, exerts a temporary, but definite, interference on the action of penicillin. This interference is conditioned by the sequence of administration and the time elapsing between injections of the antibiotics. The interference is not made apparent in survival experiments if one considers the combined use of the antibiotics without regard for optimal conditions necessary for maximal efficiency. Optimal conditions concerning the use of these antibiotics entails the administration of aureomycin four hours after the administration of penicillin. This observed interference does not contraindicate the use of aureomycin and penicillin in combined therapy, but the simultaneous use of these antibiotics does diminish the possibility of realizing the full potential of such a combination.

VACCINATION AS A PROVOKING FACTOR IN POLIOMYELITIS: AN EXPERIMENTAL APPROACH

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These studies were designed as an experimental approach to the question of whether the balance between clinical and subclinical poliomyelitis might be tipped in favor of the former by the "trauma" associated with vaccination. They were stimulated by several reports that in children there may be an association between prophylactic injection of certain bacterial antigens and subsequent development of paralytic poliomyelitis.[1–5]

Effect of vaccination (intramuscular and subcutaneous) on monkeys inoculated intravenously with virus

These experiments were carried out by vaccinating rhesus monkeys (*Macaca mulatta*) with pertussis (Lederle) vaccine alone (exp. 1) or with pertussis-diptheria-tetanus combined (Squibb) vaccine (exp. 2). The Y-SK strain of poliomyelitis virus was used in the form of monkey spinal cord.

In the first experiment, 1 ml vaccine was given intramuscularly and subcutaneously on days 1 and 2 in the right leg, followed on day 2 by an intravenous inoculation into the left leg of 2 ml of 10^{-1} cord suspension containing about 100 ID_{50} intracerebral doses of virus per ml.

In the second experiment, vaccine was only given on day 1, 2 ml intramuscularly and 2 ml subcutaneously. This was followed by virus on day 2 with an intravenous inoculation of 2 ml of 10^{-2} cord suspension containing about 10 ID_{50} intracerebral doses of virus per ml. The results are shown in table 1.

TABLE 1.—*Effect of vaccination on experimental poliomyelitis induced by intravenous inoculation of virus.*

	Vaccine	Virus	Result*
Exp. 1	1 ml on days 1 & 2	2 ml 10^{-1} on day 2	2/2
	Saline control	2 ml 10^{-1} on day 2	1/4
Exp. 2	4 ml on day 1	2 ml 10^{-2} on day 2	2/5
	Saline control	2 ml 10^{-2} on day 2	1/5
Totals	Vaccinated		4/7
	Saline controls		2/9

* 2/2 indicates that of 2 monkeys inoculated, both developed paralytic poliomyelitis.

Paralysis was not selective to the limb inoculated with vaccine. Those animals receiving the 10^{-1} suspension of infected cord and developing paralysis were found to have developed neutralizing antibodies by the first day of perceptible disease. The animals which remained well also developed antibodies when tested four weeks after inoculation. These antibody determinations were carried out in mice using the Lansing strain, which is antigenically related to the Y-SK. The procedure and typical results with monkey serums have previously been described in some detail.[6]

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TABLE 2.—*Failure of vaccination to influence poliomyelitis in cynomolgus monkeys orally infected.*

	Number of monkeys	Days																Result
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Exp. I																				
V = Vaccine: Lederle pertussis, 0.6 ml IM.+0.4 ml SQ.	5		V	P	P	P														0/5
P = Poliomyelitis virus, 10% cord	4		P	P	V															1/4
Y-SK, 3 ml per day.	5		P	P	P															0/5
Exp. II																				
V = Squibb combined pertussis, diphtheria, tetanus, 1 ml IM.	2	V	V					P	P	PV	P	V			V				V	1/2
P = Poliomyelitis virus, 10%	2							P	P	PV	P	V			V				V	2/2 } 3/4
Y-SK, 7 ml per day.	8							P	P	P	P	V								6/8 ,
Exp. III																				
V = Lederle pertussis + National diphtheria, 1 ml IM.	6	V						V	P	P	P	P							V	3/6
P = Poliomyelitis virus, 20%	8									P	P	P	P							4/8
Y-SK, 3 ml per day.																				
Totals: Vaccinated animals fed poliomyelitis virus																				7/19
Nonvaccinated controls fed poliomyelitis virus																				10/21

IM, intramuscular. SQ, subcutaneous. All inoculations were made in the upper part of the right arm.

In view of the earlier literature, it was surprising to find even two of nine control animals paralyzed after receiving these relatively small doses of virus intravenously. There was no striking difference in this group and the four of seven vaccinated monkeys. It is difficult to believe that the saline inoculation of the control animals had any bearing on the outcome. The number of experimental animals was too small for statistical evaluation.

Experiments in cynomolgus monkeys fed virus

Three experiments were carried out with *Macaca cynomolgus* (*M. irus*) in which the temporal relationships of vaccination to time of virus feeding were varied. These are indicated in table 2. Of 19 monkeys vaccinated in the upper part of the right arm before and/or after the virus feeding, seven developed the paralytic disease. Of 21 control monkeys which were fed virus but not vaccinated, ten developed paralytic poliomyelitis. Not only was there no difference in incidence of poliomyelitis in the two groups, but the vaccinated animals did not preferentially develop paralysis in the vaccinated limb.

Vaccination experiments in chimpanzees

Because poliomyelitis in chimpanzees appears to be the closest analogue to the disease in man, two experiments were carried out in these animals.

In the first, four animals were fed Y-SK virus on two consecutive days, as previously described.[7] Then on the 3rd, 6th, 9th, and 23rd days, two of the chimpanzees were inoculated in the right biceps or triceps with 1 ml of pertussis vaccine combined with diphtheria-tetanus toxoids (Squibb). There

7. Melnick, J. L. and Horstmann, D. M. 1947, J. Exper. Med. **85**: 287–303.

were no evidences of clinical poliomyelitis in any of the four animals. However, in accordance with past experience of this laboratory, all four animals were subclinically infected, as shown by the development of antibodies to the Y-SK strain six weeks after the virus feeding.

In view of clinical observations that most of the postvaccination poliomyelitis cases were grouped around a period of nine to 18 days after vaccination, Burnet[8] has suggested that the inflammatory response around the vaccine might involve small nerves or their terminals, producing a change in the nerve cells in the corresponding region of the spinal cord and rendering them highly vulnerable to infection with poliomyelitis virus. A second experiment was therefore carried out with the antigenically unrelated Brunhilde strain, in which two animals were inoculated with pertussis vaccine (1 ml of Lederle plus 1 ml of National Drug) in the left biceps. The pertussis vaccination was repeated on the seventh day. On the eighth, ninth, and tenth days Brunhilde virus was administered to all four animals. On the 18th day, the first two chimpanzees received another inoculation of the vaccine. Again no animal developed any clinical signs of poliomyelitis.

Experiments with Lansing poliomyelitis virus in mice

Experiments were carried out to determine whether pertussis vaccination could influence the disease in mice by increasing either the titer or the peripheral invasiveness of the virus. With pertussis vaccine (Squibb) inoculated intraperitoneally (0.2 ml) and intramuscularly (0.1 ml in right leg), it was found that: (1) The intracerebral titer of Lansing virus was not influenced when mice were inoculated one day before and nine days after with pertussis vaccine. (2) Lansing virus did not produce paralysis when inoculated intraperitoneally or intramuscularly into mice vaccinated with pertussis simultaneously, previously, or subsequently. (3) Inoculation of Lansing virus intracerebrally into mice vaccinated in the muscle of the right leg did not increase the incidence of paralysis in this limb. This was true regardless of whether mice received 1, 10, or 100 ID_{50} doses of virus.

There was no evidence from our experiments that the interval between the injection of virus and the onset of paralysis was shorter in mice previously inoculated with pertussis vaccine. This is contrary to the reports of Findlay[9] and Milzer,[10] who, however, in their work, used different antigen preparations. Also, in one laboratory[9] a different route of antigen administration was used, and in the second,[10] different temporal relationships between virus and vaccine administrations.

Experiments in mice with spontaneous encephalomyelitis virus (Theiler's TO virus)

Among the explanations that can be offered for the fact that a higher incidence of paralytic poliomyelitis may occur in children vaccinated against pertussis, is that the vaccine had activated latent poliomyelitis virus in these children. Since it had been demonstrated that latent herpes simplex encephalitis in rabbits can be activated by anaphylactic shock,[11] it seemed pertinent to de-

8. Burnet, F. M. 1950, cited in editorial, Brit. Med. J. 1: 1004–1005.

9. Findlay, G. M. and Howard, E. M. 1950, J. Path. Bact. **42**: 371–382.

10. Milzer, A., Weiss, M. A. and Vanderboom, K. 1951, Proc. Soc. Exper. Biol. & Med. **77**: 485–488.

11. Good, R. A. and Campbell, B. 1948, Proc. Soc. Exper. Biol. & Med. **68**: 82–87.

termine the effect of a sensitized state induced either by pertussis or horse serum injections on latent Theiler's encephalomyelitis of mice.

For these experiments four- to 5-week-old mice proven to be intestinal carriers of virus were used.[12] One group of 30 mice received repeated intraperitoneal injections of pertussis vaccine (Squibb, 0.5 ml), and as a control a second group of 24 mice received normal horse serum (0.5 ml). Each animal received four infections, one every second day. After a rest period of one week, the animals were challenged, each group with its own vaccine. Some animals died of anaphylactic shock in both groups shortly after the injection. Of the surviving mice which had received the pertussis vaccine, eight were tested for sensitivity to histamine,[13] and five of the eight mice proved to be sensitive. Just before the challenge was given, feces were collected from all the mice, and virus was again found in both of the groups. In spite of the fact that these mice were proven to be latent carriers of virus and to be hypersensitive to the vaccine used, there was no effect on the incidence of clinical myelitis. None of the mice developed paralysis in the ensuing observation period of one month.

12. Theiler, M. 1941, Medicine, **20**: 443–462.
13. Parfentjev, I. A. and Goodline, A. 1948, J. Pharmacol & Exper. Therap. **92**: 411–413.

CONCLUSIONS

In the present experiments on chimpanzees, monkeys, and mice, the occurrence of paralysis was not enhanced by the vaccination procedure. Our negative results in these experiments devised to demonstrate the relationship of vaccination and poliomyelitis do not discredit the possibility of such an association. Other mechanisms than those which we have explored here may underlie the observations made in man.

ACQUIRED RESISTANCE IN *CANDIDA ALBICANS* TO AN ANTIBIOTIC PRODUCED BY A SPOREFORMING BACILLUS

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The pattern of resistance of bacteria to antibiotics has aroused much interest. Resistance in fungi, however, has not been studied extensively, possibly because of the limited number of antibiotics possessing antifungal activity. The research presented in this paper was undertaken to find whether the patterns of resistance which may exist in the fungi have any relation to those known for bacteria.

With streptomycin and penicillin the change from sensitivity to resistance is considered to be due to mutation, the antibiotics acting as selective agents eliminating the sensitive forms and allowing the resistant mutants to multiply (Demerec, 1949). The pattern of increase in resistance is characteristic for the antibiotic and not for the specific organism. Thus, for penicillin the first-step mutants are fairly uniform in degree of increase and only slightly higher in resistance than the original strain. Demerec (1949) has stated that at least two mutations in the same organism are necessary for a significant increase in resistance and that the chances of this occurring are only 1×10^{-16} per bacterium per generation, since the mutation rate is low, about 1×10^{-8} per bacterium per generation. The genes for resistance to penicillin have a similar potency, so that the mutation of each gene gives a stepwise, slightly increased resistance.

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With streptomycin the variation in resistance gained by first-step mutants is very great, being all the way from slight to high resistance in one mutation step. From this one may infer that the genes vary greatly in potency and that the mutation of a high potency gene may give complete resistance in one step. Newcombe and Hawirko (1949) gave the mutation rate for streptomycin resistance in *Escherichia coli* as 1×10^{-10} per bacterium per division cycle. English and McCoy (1951) gave a value in the same general range, 1 to 6.7×10^{-9}, for *Micrococcus pyogenes* var. *aureus*. Resistance to streptomycin and to penicillin is typical of mutation in that it is permanent during serial transfer in nonantibiotic mediums (Murray et al, 1947; Bellamy and Klimek, 1948).

Resistance of *M. pyogenes* var. *aureus* to bacitracin rises steadily with serial contact with the antibiotic and tends to level off at a titer about 50 to 60 times that of the original (Stone, 1949). Resistance, however, is not permanent, as is shown by a rapid loss of resistance during daily transfer in bacitracin-free medium.

MATERIALS AND METHODS

The antibiotic used for these experiments is produced by bacillus B-40, resembling *Bacillus subtilis*, and is active against both gram-positive and -negative bacteria and against several of the yeasts and yeast-like fungi, including *Candida albicans*. The strain of *C. albicans* used came from Dr. L. J. Wickerham of the Northern Regional Research Laboratory and is carried by him under the strain number 477.

Antibiotic broths were produced in shake flask cultures by bacillus B-40 in a glucose, corn steep, soyabean meal, carbonate medium. At age of 15 to 24 hours the broths were adjusted to pH 4,

steamed, centrifuged and the supernatant filtered through a sterile Seitz filter. For the work reported here this crude preparation was satisfactory. Further work in purification of the antibiotic is being done, however, both by the writer and by Mr. John W. Daniel, Jr., of the Biochemistry Department of this university.

A dilution assay with cultures in glucose 1%, yeast extract 0.5% broth at pH 4 was used, because *C. albicans* grows well and the antibiotic is stable at this pH. Tubes containing 5 ml of the above broth are sterilized. Five ml of the sterile antibiotic sample are added to 5 ml of assay broth (both at pH 4) and the contents shaken. Five ml of this mixture are then added to a second 5 ml assay broth and the contents shaken. This procedure is repeated so as to give dilutions of the original antibiotic sample of 1:2, 1:4, 1:8, etc., up to 1:2048. In the range of dilutions between 1:100 and 1:1000 the difference between antibiotic concentrations in the tubes becomes sufficiently large so that three additional dilutions are added to the series for greater precision of the assay. An aliquot of 0.05 ml of the 1:4 dilution is added to 5 ml of assay broth to give a dilution of approximately 1:400, and similarly 0.05 ml of the 1:8 dilution is added to 5 ml assay broth to give approximately a 1:800 dilution. A dilution of about 1:700 is obtained by adding 0.5 ml of the 1:64 dilution to 5 ml of assay broth.

The dilution tubes are inoculated with a standard loop from an 18-hour stationary culture of *C. albicans* and then incubated at 37 C for 48 hours. Readings are taken at both 24 and 48 hours for those tubes which show complete inhibition of growth of the organism. Thus the dilution unit is the least concentration of the antibiotic which will still inhibit growth. For lack of a purified product on which to base a unit by weight, the dilution unit for complete inhibition of growth at the above time of reading had to be used in the present work.

EXPERIMENTAL RESULTS

When readings of the antibiotic assay tubes are taken every 24 hours, a phenomenon of shifting end point is noted. Growth appears, on successive readings, in the presence of greater and greater concentrations of the antibiotic; see figure 1, data for the sensitive strain. If the logarithms of the dilution units of antibiotic are plotted against days of incubation, a straight line is obtained. In other words, in the presence of increasing concentrations of the antibiotic the lag period of growth is also increased. Filtrates of the 48-hour dilution assay tubes, if reassayed, show the same antibiotic activity as in the original assay. Thus destruction of the antibiotic cannot explain the secondary growth in the later readings.

Resistant strains of *C. albicans* were obtained by growing the organism in the presence of the antibiotic at two levels, i.e., at maximum antibiotic concentration which would allow slight growth and also at a concentration well below that required for inhibition. A dilution series from an antibiotic preparation which showed an activity of 500 dilution units at 24 hours was incubated at 37 C for 72 hours. At this time growth appeared in all tubes except the 1:2 dilution tube. The 1:4 dilution tube was then used as the inoculum for another series, with the same antibiotic preparation. At the end of 48 hours of incubation growth appeared in all of the tubes with 1:8 or greater dilution. The 1:16 dilution tube of this series was again used for inoculation of a series. At the end of 48 hours of incubation growth had appeared in all but the 1:2 dilution tube. The 1:4 dilution tube was subcultured onto agar slants containing no antibiotic, and after 24 hours of incubation these were stored in a refrigerator and used as stock cultures of possible resistant *C. albicans* strains. These results are summarized in figure 1.

Resistant cultures were also obtained by subculturing from those tubes in the dilution series in which the antibiotic concentration was much less than that required for inhibition. In an antibiotic dilution series in which the inhibitory level was at a dilution of 1:128 at 48 hours, the 1:1000 dilution tube

Dilution	*Series 1 Readings*		*Series 2 Readings*	*Series 3 Readings*	
	24 hr.	*48 hr.*	*48 hr.*	*48 hr.*	
1:2	−	−	−	−	Resistant
1:4	−	+	−	+	→stock
1:8	−	+	+	+	
1:16	−	+	+	+	
1:32	−	+	+	+	
1:64	−	+	+	+	
1:128	−	+	+	+	
1:256	−	+	+	+	
1:512	−	+	+	+	
1:1024	+	+	+	+	

FIG. 1.—Scheme for deriving strains of *C. albicans* that show resistance to antibiotic B-40.

was used for inoculum for another series which used the same stock antibiotic preparation. This second set of dilution tubes gave growth at a dilution of 1:4 at 24 hours of incubation and maximum growth by 48 hours.

Since the time of reading the dilution assay is fixed at 24 hours and 48 hours after inoculation, a striking difference is found in the concentration at which the two forms are inhibited at these particular times. This is illustrated in figure 2, in which the resistant and sensitive strain have both been allowed to grow in the presence of dilutions of the antibiotic up to approximately 2000 units. By 48 hours growth had occurred in all the dilution tubes in which the resistant strain had been inoculated and only in the 1:2050 tube of the sensitive culture. If the dilution tubes are incubated long enough so that secondary growth arises in the sensitive culture, for example after 6 days in the presence of 2000 units of antibiotic per ml (see fig. 2), a subculture of it will show that the secondary growth is, in fact, resistant.

The pattern of growth of a resistant culture is illustrated in figure 2, in which is shown the growth of the sensitive and resistant strains in the presence of antibiotic in dilutions from 1:2 to 1:2000. The graph for the resistant culture is the same, no matter in which way the resistant strain is derived.

Resistance in *C. albicans* may, then, be defined as the ability of the organism to grow more quickly in the presence of high concentrations of antibiotic than does the original culture. From this it is inferred that the action of the antibiotic

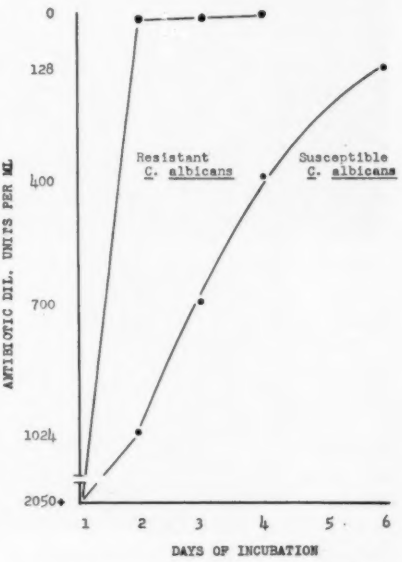


FIG. 2.—A comparison of the growth of an antibiotic-resistant and an antibiotic-susceptible strain of *C. albicans* in the presence of approximately 2000 dilution units of antibiotic.

is largely fungistatic but not entirely so. In the stock-sensitive strain about 25 to 35% of the cells are killed by a concentration such as 128 units of the antibiotic per ml. This was shown by letting cells stand with the antibiotic for two hours, then diluting out the suspension and making plate counts.

This antibiotic resistance in *C. albicans* is relatively permanent but not completely so, as was shown by continuous subculture in absence of the antibiotic every 24 hours on agar slants at 37 C until approximately 40 transfers had been made. The final culture was able to grown in the presence of a 1:32 dilution of the antibiotic stock preparation within 48 hours of incubation. The original resistant culture grew in a 1:2 dilution with 48 hours of incubation. Thus the transferred culture lost resistance by a factor of 16-fold in a culture whose original resistance was 64 times that of the susceptible control.

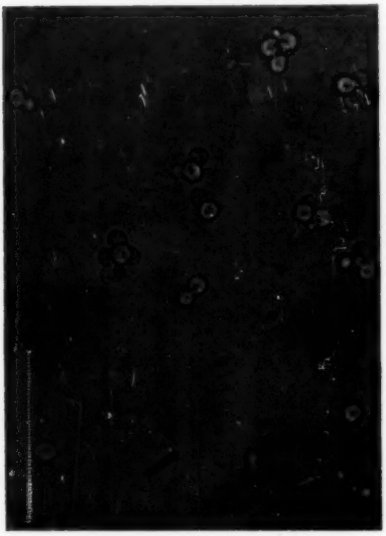
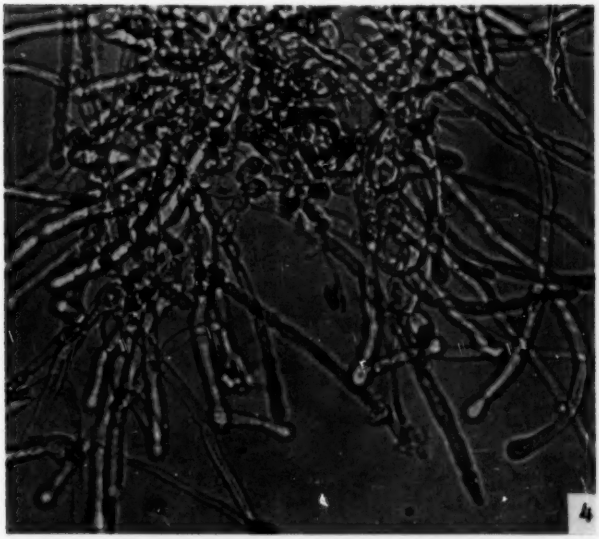


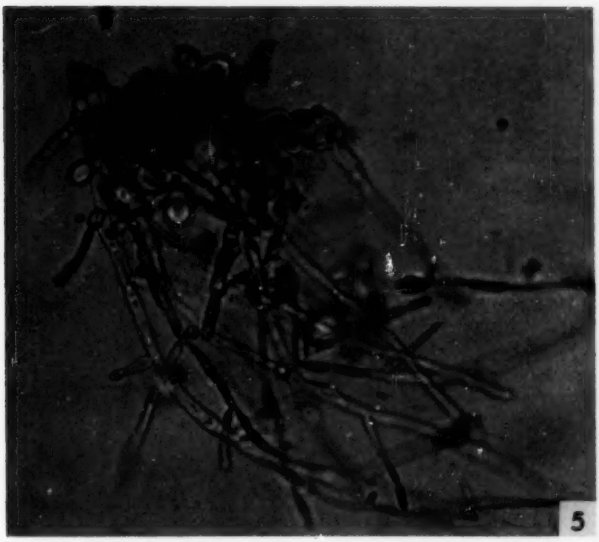
FIG. 3.—Yeast phase growth of *C. albicans*.

Morphological variations accompanying resistance

Cultures of *C. albicans* which are able to grow in the presence of this antibiotic undergo morphological variation both in cellular and colony form. Cultures used for inoculum of the antibiotic dilution assay are largely in the yeast phase (it will be remembered that *C. albicans* may exist in either yeast or mycelial phase). Those cells that are able to grow in the presence of the antibiotic are converted to the mycelial phase, concurrent with the acquired resistance to the antibiotic. The yeast phase of growth is shown in figure 3, and the mycelial phase induced by the antibiotic in figures 4 and 5. The mycelium grows as balls of cells in the bottom of the liquid. It resembles somewhat pellets of mold grown in shake flask culture. It is typical of the literature description of *C. albicans* in the mycelial phase, except somewhat lacking in blastospores. In nonantibiotic broth the sensitive strain grows in a yeast phase, although in week-old stationary cultures a mat of submerged mycelium may form, which breaks up readily upon shaking and liberates blastospores. The resistant strain under these conditions forms a firm interwoven mat of submerged mycelium that does not break up upon shaking.

Giant colonies of the resistant strain on Sabouraud's agar differ strikingly from the control, and they resemble somewhat the dissociated cultures described by Mackinnon (1940), who found them occurring naturally, and by Mickle and Jones (1940), who found them by growing *C. albicans* serially in Sabouraud's broth with 0.25% lithium chloride. Figure 6 shows the control sensitive culture and figure 7 the resistant form at age 7 days. The old mycelial colony is rather hard and dry and can easily be lifted intact from the agar. It is made up of interwoven mycelium, lack-

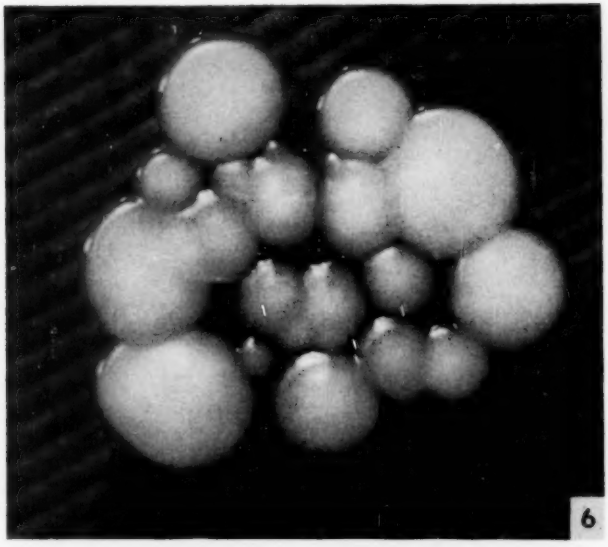




FIGS. 4 and 5.—Mycelial phase growth induced by the antibiotic.

ing blastospores. The typical forms of the colonies hold for the sensitive and the resistant cultures, whether the cultures are plated on Sabouraud's agar with or without the antibiotic, but the roughness is enhanced by even subinhibitory levels of the antibiotic.

An experiment was set up to test



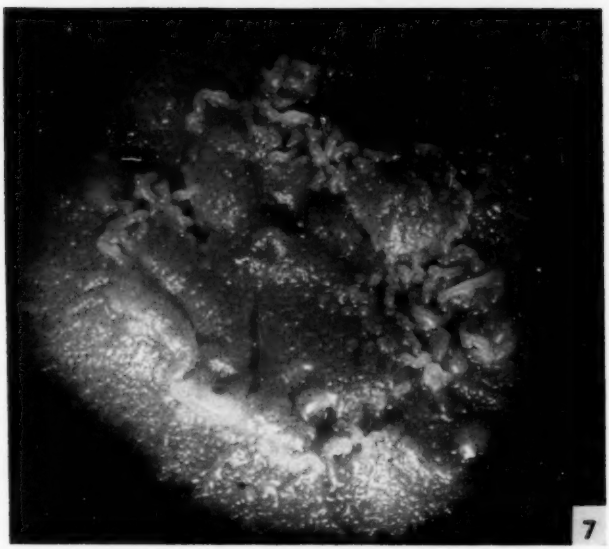


FIG. 6.—Giant colonies of control, antibiotic-sensitive strain of *C. albicans*.
FIG. 7.—Giant colony of antibiotic-resistant strain of *C. albicans*.

whether cultures dissociated by lithium chloride by the method of Mickle and Jones (1940) would acquire resistance to the antibiotic as they acquire the mycelial phase. Broth, as used in antibiotic dilution assays, containing 0.25% lithi-

um chloride was adjusted to pH 4 and inoculated with the sensitive *C. albicans* culture. This culture was incubated 24 hours at 37 C and then subcultured to fresh medium of the same type. After 36 such transfers the culture was assayed against the antibiotic in the absence of lithium chloride and was found to have an increase in resistance to the antibiotic four times that of a stock culture run concurrently in plain medium. The inoculum used for this assay was also grown in the absence of lithium chloride. These data appear in table 1.

Lithium chloride in the assay broth itself also decreases the sensitivity of *C. albicans* to the antibiotic, on the part of either sensitive or resistant cultures. Also, the culture which has been previously serially transferred in lithium chloride broth shows the same sensitivity to the antibiotic whether lithium chloride is or is not present in the assay broth. These data also appear in table 1.

From the experimental results presented it would appear that dissociation of *C. albicans*, whether induced by the antibiotic or by lithium chloride, leads to a state of resistance to the antibiotic. Since dissociated strains of *C. albicans* have been reported to be less virulent at least for rabbits (Mackinnon, 1940; Mickle and Jones, 1940), perhaps an antibiotic such as B-40 may be of value in dissociating the pathogen to a less virulent form with which the body can then contend.

TABLE 1.—*Effect of lithium chloride on antibiotic resistance.*

Culture	Assay in normal broth		Assay in LiCl broth	
	24 hr.	48 hr.	24 hr.	48 hr.
Stock—not grown with LiCl				
Sensitive	1:128*	1.32	1:32	1:8
Resistant	1:16	1:2	1:2	1:2
After 36 transfers in 0.25% LiCl broth	1:32	1:8	1:32	1:8

* Highest dilution giving complete inhibition.

SUMMARY

Studies are presented on the resistance developed by *Candida albicans* to a new antifungal antibiotic produced by a bacillus B-40, resembling *Bacillus subtilis*. This resistance manifests itself as a decrease in the lag period before *C. albicans* is able to grow in the presence of the antibiotic. The lag period varies with the logarithmic function of antibiotic concentration, and the antibiotic is not destroyed during growth. Only a fraction of the cells are killed outright by the antibiotic.

Strains of *C. albicans* resistant to this antibiotic can be easily developed by subculturing from the greatest antibiotic concentration at which growth will occur or by subculturing in series from growth occurring at levels of antibiotic well below the inhibitory level. After transfer 40 times in the absence of antibiotic, a resistant culture shows a partial loss, i.e., 16 times in a culture whose original resistance was 64 times that of the sensitive parent.

Resistance to the antibiotic is accompanied by conversion of the normal yeast phase of growth of *C. albicans* to strongly mycelial growth, both in broth culture and in giant colonies on agar. This morphological variation resembles that which occurs in dissociated strains of *C. albicans* both at random in a normal population and during serial transfer in lithium chloride broth. Cultures dissociated by the latter procedure acquired moderate resistance to the antibiotic.

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THE CHANGING PICTURE OF ENCEPHALITIS IN THE YAKIMA VALLEY, WASHINGTON

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During the past ten years the epidemiology of the arthropod-borne viral encephalitides has been studied intensively in a number of geographical areas. The Yakima Valley of Washington was the first of these areas from which sufficient information became available to allow an understanding of some of the principal epidemiological factors which lead to infection of the human and equine hosts.

The early studies in this area were carried out during the epidemic years of 1940,[1,2] 1941,[3] and 1942.[4] Here Western equine and St. Louis viruses first were isolated from naturally infected mosquitoes.[5] During the succeeding years of 1943 through 1950 epidemics did not recur, yet a study made in 1944[6] provided evidence of high virus infection rates in *Culex tarsalis* Coquillett, indicating that both Western equine and St. Louis viruses were at a hyperendemic level in their natural cycles in the lower animals.

In spite of the ready availability of the infectious agents in the vector in 1944, the number of recognized clinical cases in man or horse was minimal. A

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plausible explanation, at least partially supported by evidence, was that the extensively exposed resident human and equine populations had been immunized through inapparent infection. As a result, the probability of occurrence of clinical infection had been brought down to a very low level. That such a phenomenon would not occur in the avian hosts was to be expected because a large number of susceptible birds is hatched every year and the life of these hosts is relatively short. These two factors combined to prevent a material accumulation of immune birds.

During this latent period (through 1944), there had been no observable alteration in the abundance of mosquito vectors, or of vertebrate hosts, or in environmental factors. Any of these alterations would have served as an explanation of the changed epidemiological picture.

In 1948 circumstantial evidence came to our attention indicating a change in one factor probably affecting the epidemiology of encephalitis in this area: a marked decline in vector population. Correspondence from persons in the Yakima Valley pointed out that the widespread use of the new insecticides, principally DDT, on agricultural crops had cut down the mosquito population noticeably. This modified situation was believed by us to offer an opportunity for evaluating the possible control of the arthropod-borne viral encephalitides through vector control.

Investigations were conducted between August 2 and August 15, 1949. On the basis of past experience in this area, it was believed that this was likely to be the period of most active vector transmission of the encephalitis viruses.

The incidence of clinical infection in man and horse in recent years was studied, and blood specimens were collected to determine the incidence of neutralizing antibodies to the encephalitis viruses in wild and domestic avian hosts. Plans to collect blood specimens from the human population unfortunately were not successfully completed. Possible mosquito and mite vectors were collected to determine the prevalence of viruses in these arthropod vectors.

In the following sections evidence is presented that a change had taken place in the environment and vectors and the immunological status of avian hosts, influencing the epidemiology of the viral encephalitides in the Yakima Valley.

TABLE 1.—*Yearly incidence of clinically recognized cases of encephalitis in the Yakima Valley, Washington during the summer months (May through September, 1938–1949).**

Year	Number of cases	Number of deaths	Case fatality rate, percent
1938	4	Unknown	
1939	31	Unknown	
1940	58	13	22.4
1941	28	3	10.7
1942	28	2	7.2
1943	1†	1	
1944	5	5	
1945	3	3	
			53.8
1946	14	4	
1947	0	0	
1948	2	1	
1949	1†	0	

* Based on records of the Washington State Health Dept.
† Confirmed as Western equine by laboratory tests.

Clinical and inapparent infection in vertebrate hosts

Man

Historically, four epidemics of Western equine and St. Louis encephalitis have been recognized in this area—in the summers of 1939, 1940, 1941, and 1942.[1–4] The attack rates were highest in 1940 (62.7 per 100,000) in Yakima County exclusive of Yakima City; the attack rate was 44.1 per 100,000 in the city. Since 1942 relatively few clinical cases have been recognized as possible representatives of this group of infections (table 1). Of the 26 cases re-

ported,[7] the diagnoses in only two were confirmed by laboratory tests, and these were of the Western equine type. Serological tests were made in a few of the remaining 24 cases, but the number tested and the results obtained are not available to us. During this seven-year period (1943–1949), in only one year, 1946, did the number of cases approach epidemic proportions.

In the period 1943 through 1949 the case fatality rate was unusually high—53.8% (table 1). The case fatality rate for the epidemic period 1940 through 1942 was only 15.8%. This apparent sharp rise might well be attributed to failure to report mild cases or to misdiagnosis of a number of cases of encephalitis. In spite of these possibilities, it must be concluded that the incidence of clinically recognized encephalitis dropped markedly in this area.

As previously mentioned, a possible explanation of this decrease in the incidence of clinical infection is the immunization of a substantial proportion of the population as a result of subclinical infection. There is some evidence in earlier studies to support this idea. In 1941[3] and 1942,[4] 51.9% of the serums obtained in this area from over 100 residents with no history of clinical encephalitis contained neutralizing antibodies to the St. Louis virus. At the same time only 3.9% of 127 residents had antibodies to the Western equine virus. Yet in 1944,[6] 29.3% of serums from 24 normal persons had Western equine antibodies; these were not tested against St. Louis virus. Unfortunately, these figures cannot be adjusted by available data for duration of residence. Significance, therefore, is somewhat questionable. A high proportion of positive serologic reactions in a population, together with a low clinical attack rate, apparently characteristic of these viruses, would be expected to lead to the occurrence of very few clinical cases, even though the infectious agents were commonly present in the natural hosts and vectors. This might be advanced as one of the cardinal characteristics of the epidemiology of these two encephalitides in most areas where they have been established for some time. In a stable population, it would be reflected in a low age distribution of the observed cases, such as occurs in Japanese B encephalitis in Japan and Okinawa.[8]

In the period 1940 to 1950, the population of Yakima County rose 36.5% and the population of the city of Yakima increased 41%.[9] One could expect that many immigrants to this area came from nonendemic areas and would be susceptible, as has been observed of immigrants into another endemic area, Kern County, California.[10] The rise in population due to resident births might be expected to include a high proportion of children of immune mothers. These children would be protected temporarily by maternal antibodies. Unfortunately, data are not available on precisely when the population grew, but probably the expansion was greatest in the postwar period, the same time in which the mosquito factor in the infection chain began to be affected, as will be discussed later. A study of the immunity status of persons who were residents of the Valley at the time of population growth might have clarified this problem.

At the time of this study the age distribution of reported cases (table 2)

7. Records of Washington State Health Dept.

8. Tigertt, W. D. and Hammon, W. McD. 1950, Japanese B encephalitis: a complete review of experience on Okinawa 1945–1949. Am. J. Trop. Med. **30**: 689–722.

9. U. S. Census Report, 1950.

10. Hammon, W. McD. and Reeves, W. C. Unpublished data.

TABLE 2.—*Age distribution of reported cases of encephalitis, Yakima Valley, Washington.*

Age group	1940	1941	1942	1943–1949
0– 4	4	8	5	3
5– 9	2	3	1	1
10–14	0	0	3	1
15–19	3	2	0	1
20–29	6	7	2	3
30–39	7	1	2	2
40–49	5	2	5	3
50–59	11	1	2	5
60+	20	4	7	5
Unknown	0	0	1	1
Totals	58	28	28	25
Mean age	46	24	36	39

had not shifted materially from that noted in earlier years:[1–4] the mean age is 39 years; it had been 46 for 1940, 24 for 1941, and 36 for 1942. This does not clarify the immunity status of the population, as no information was available on how long those with clinical infections had been residents of the area.

In the early years of epidemic encephalitis in this area, many of the patients were susceptible migratory fruit pickers. A considerable decrease in migratory labor in later years might have lowered significantly the number of susceptible persons working at an occupation where they were most likely to be exposed to infection.

Horses

Clinically recognized infection in horses has closely paralleled that in man. A conspicuous epizootic broke out in 1939, when it was estimated that there were over 500 cases of equine encephalomyelitis.[6] In succeeding years, the number of reported cases has progressively diminished. In 1949 only three suspected cases were encountered by veterinarians. The reported number has decreased in spite of the fact that more veterinarians are practicing in the area, thus enhancing the chances of cases being observed. The horse population has remained relatively constant during the past ten years. At the same time, the proportion of horses vaccinated against Western equine encephalitis has decreased each year. The local veterinarians agreed that in 1949 less than 1000 of the estimated 4500 horses in the Yakima Valley had been vaccinated.

In 1941, 100% of a sample of 18 normal unvaccinated horses had serum neutralizing antibodies to the Western equine virus, and 85% of 27 had neutralizing antibodies to the St. Louis virus.[11]

It appears that accumulated immunization, largely due to inapparent infection, has played a major role in cutting down the clinical attack rate in horses. Once again, as in man, this might be considered an epidemiological characteristic of the Western equine infection in horses in an endemic area.

Birds

It is generally accepted that birds are of primary importance as the principal vertebrate hosts of Western equine and St. Louis viruses. The infection in birds is typically an asymptomatic, transient infection with a viremia of short duration, followed by rapid development of serum neutralizing antibodies.[12–14] During viremia, birds serve as a source of

11. Hammon, W. McD., Lundy, H. W., Gray, J. A., Evans, F. C., Bang, F. and Izumi, E. M. 1942, A large-scale serum neutralization survey of certain vertebrates as part of an epidemiological study of encephalitis of the Western equine and St. Louis types. J. Immunol. **44**: 75–86.
12. Hammon, W. McD. and Reeves, W. C. 1946, Western equine encephalomyelitis virus in the blood of experimentally inoculated chickens, J. Exper. Med. **83**: 163–173.
13. Hammon, W. McD., Reeves, W. C. and Izumi, E. M. 1946, St. Louis encephalitis virus in the blood of experimentally inoculated fowls and mammals. J. Exper. Med. **83**: 175–183.
14. Hammon, W. McD., Reeves, W. C. and Sather, G. E. 1951, Western equine and St. Louis encephalitis viruses in the blood of experimentally infected wild birds and epidemiological implications of findings. J. Immunol. **67**: 357–367.

vector infection. In a study of the epidemiology of these infections, the prevalence of serum neutralizing antibodies is a most useful index of the current activity of these viruses in an area.

During 1941, a large-scale serum neutralization survey was made of Yakima Valley birds.[11] The incidence of Western equine and St. Louis antibodies was high. In 1949, a series of blood specimens was obtained from the same species of birds. It should be emphasized that in the 1949 series the majority of birds sampled were less than one year of age, whereas in the previous study all age groups were included. The results of neutralization tests on these blood specimens are presented in table 3.

There was very little serologic evidence of Western equine virus infection during 1949. Only 1% of the specimens tested were positive, as compared with 24.5% in 1941 (table 4). It is interesting to note that the three species of birds that had Western equine antibodies—Brewer blackbird, killdeer, and robin—are all migratory species. Purely resident species such as the chicken, pigeon, California Valley quail, Chinese ring-necked pheasant, and English sparrow gave no perceptible evidence of previous infection; the first four of these had had a high proportion of positive serologic reactions in earlier studies. These observations are interpreted as an indication of an extremely low level of Western equine virus transmission.

TABLE 3.—*Neutralization tests on Yakima bird blood serums collected in August, 1949.*

Species	Western equine				St. Louis			
	+*	±†	0	Total	+*	±†	0	Total
English sparrow	0	5	63	68	1	3	51	55
Song sparrow	0	0	23	23	0	0	7	7
Brewer blackbird	1	5	22	28	2	2	21	25
Killdeer	1	2	19	22	3	1	16	20
Robin	1	2	14	17	6	2	8	16
Mourning dove	0	3	18	21	2	1	16	19
Chinese ring-necked pheasant	0	3	13	16	3	2	10	15
California Valley quail	0	1	9	10	1	0	9	10
Pigeon	0	0	4	4	0	0	4	4
Wilson snipe	0	0	4	4	1	0	3	4
Mallard duck	0	0	5	5	0	0	5	5
Redwinged blackbird	0	0	4	4	1	1	2	4
Hungarian partridge	0	2	3	5	1		4	5
Bank swallow	0	0	4	4	0	2	1	3
Barn swallow	0	0	1	1	0	1	0	1
Meadow lark	0	1	3	4	1	0	3	4
Eastern kingbird	0	1	1	2	0		2	2
Green winged teal	0		2	2	0		2	2
Kingfisher	0		1	1	0		1	1
Pacific nighthawk	0		1	1	0		0	0
Shrike	0		1	1	1		0	1
Magpie	0		1	1	1		0	1
Housefinch	0		2	2	0		1	1
Chicken‡	0	3	53	56	0	0	55	55
Totals	3	28	271	302	24	15	221	260

* Positive indicates a neutralization index of over 10.
† Equivocal indicates a neutralization index of 5 to 10.
‡ Collected in November, 1949.

TABLE 4.—*Comparison of neutralization test findings on serums of Yakima Valley birds collected in 1941 and 1949.*

Species	Western equine virus				St. Louis virus			
	1941		1949		1941		1949	
	Number tested	Percent positive*	Number tested	Percent positive*	Number tested	Percent positive*	Number tested	Percent positive*
English sparrow	8	0.0	63	0.0	11	0.0	52	1.9
Brewer blackbird	13	0.0	23	4.4	19	0.0	23	8.7
Killdeer	7	28.6	20	5.0	9	0.0	19	15.8
Robin	13	46.2	15	6.7	12	58.3	14	42.9
Mourning dove	6	0.0	18	0.0	5	60.0	18	11.1
Chinese ring-necked pheasant	8	25.0	13	0.0	9	33.3	13	23.0
California Valley quail	10	30.0	9	0.0	10	30.0	10	10.0
Redwinged blackbird	5	0.0	4	0.0	3	0.0	3	33.3
Pigeon	10	50.0	4	0.0	11	63.6	4	0.0
Chicken	31	45.2	53	0.0	32	28.1	55	0.0
Miscellaneous wild species	52	15.4	52	0.0	57	25.0	34	14.7
Totals	163	24.5	274	1.1	178	25.8	245	9.8

* Percent positive calculated by excluding all equivocals from the totals; see table 3 for number of serums giving equivocal reactions.

TABLE 5.—*Species and number of adult and immature birds of the Yakima Valley from which blood smears were examined for plasmodium parasites (collected in August, 1949).*

Species	Number of birds examined		
	Adult	Immature	Total
Mallard duck	2	3	5
Wilson snipe	0	5	5
Killdeer	0	21	21
Mourning dove	11	12	23
Meadowlark	1	3	4
Redwinged blackbird	1	4	5
Brewer blackbird*	41	119	160
Song sparrow	4	31	35
English sparrow*	13	57	70
Bank swallow	2	13	15
Barn swallow	5	4	9
Robin	5	14	19
Chinese ring-necked pheasant	1	16	17
California Valley quail	2	7	9
Hungarian partridge	0	6	6
Pigeon	3	2	5
Miscellaneous species†	5	14	19
Totals	96	331	427

* One immature bird of this species was infected with *Plasmodium relictum*.

† One or two birds of each of the following species were sampled: greenwinged teal, bluewinged teal, belted kingfisher, arctic three-toed woodpecker, pacific nighthawk, western wood pewee, eastern kingkird, magpie, housefinch, vesper sparrow, sage sparrow, cassins finch, shrike, and longtailed chat.

In tests for St. Louis antibodies, 9.8% were positive, a higher proportion than was found with Western equine antibodies (table 4). However, it is considerably lower than the 25.8% positive for St. Louis antibodies found in 1941 (table 4). St. Louis antibodies were found in both resident and migratory species. These differences could be explained in at least two ways:

1. The principal mode of transmission of these two viruses may be different. The St. Louis virus may be transmitted in several different ways or it may have multiple arthropod vectors, one of these is the same as for Western equine (namely, a mosquito vector, *C. tarsalis*). The observed reduced incidence of reactions against the St. Louis virus could be the counterpart of the almost complete disappearance of Western equine antibodies. The continuing infection with St. Louis virus may represent a second vector or mechanism of transmission which Western equine virus does not have.

2. A second explanation may be that the same basic method of transmission serves for both, but that the cycle of transmission of the Western equine virus is more readily affected by a change in conditions, such as a decline in vector population.

In the present discussion, the pertinent factor is the evidence of a reduction in infection in 1949 as compared with the earlier study period.

In epidemiological studies in Kern County, California, avian malaria indices have been valuable as a measure of the frequency of mosquito feedings on wild birds.[15] For comparison, 427 blood smears from Yakima birds were examined (table 5). Only two immature birds were found infected with *Plasmodium relictum*. In contrast, in Kern County, California, avian malaria infection rates have been consistently high (from 10 to 50% positive) among most of the same species of birds. Unfortunately, a suitable series of blood smears from the Yakima Valley for an earlier period are not available for comparison.* Epidemiologically, one would expect that a decrease in vector population to a level where feedings on birds were limited would be reflected in a parallel decrease in malaria transmission and incidence of parasitemia.

Vector infection

Culex tarsalis

Previous studies indicated that *C. tarsalis* is the principal mosquito vector

* A short series of 52 blood smears from wild birds made in 1941 included three infected with *Plasmodium* sp.

15. Herman, C. M., Reeves, W. C., McClure, H. E., French, E. M. and Hammon, W. McD. Studies in avian malaria in vectors and hosts of encephalitis in Kern County, California. I. Infections in avian hosts. To be published.

of Western equine and St. Louis encephalitis viruses in the Yakima Valley.[3–6,16] During the current survey, this species was collected from August 2 to August 15, 1949. A total of 3277 females were tested for virus. Virus was not isolated, although more sensitive techniques of testing[17] were used than in earlier studies. A small sample of 175 *Culex pipiens* Linnaeus was collected, and these were also negative on virus tests.

These findings are impressive when compared with those in earlier investigations (table 6). Insofar as possible, mosquitoes were collected in the same locations as in earlier years. If the ratio of virus isolations to *C. tarsalis* tested in 1949 had been as high as in previous years, the expected number of isolations would have been 13 on the basis of previous average infection rates.

For comparative purposes, a survey was made of avian malaria infection rates in two species of *Culex*. Of 346 *C. tarsalis* dissected, 1.4% harbored *Plasmodium*, while none of 70 *C. pipiens* were infected. These rates are very low as compared with average infection rates of over 10% found in *C. tarsalis* in Kern County, California.[18] These findings, together with the previously described low incidence of plasmodium infection in birds, are believed to confirm the fact that in 1949 *C. tarsalis* was feeding relatively infrequently on avian hosts.

TABLE 6.—*Encephalitis virus isolations from* Culex tarsalis *collected in the Yakima Valley during four surveys.*

Year	Number of mosquitoes tested	Number of virus isolations	Ratio of virus isolations to mosquitoes tested*
1941	4655	8	1:582
1942	9446	45	1:210
1944	1967	10	1:197
1949	3277	0	0:3277

* Since these were tested in pools of various sizes, usually about 75 specimens, this represents a minimal infection rate, postulating only one infected specimen in a positive pool.

This leads to the question of why these changes took place.

Vector population

Mosquitoes

Our earlier studies on vector populations were available for comparison with current data. The mosquitoes of the area (19 species) and their ecology were known, and maps of permanent breeding areas were available. Earlier the mosquito population had been very large, as is expected in an agricultural area where an unlimited supply of cheap water is used for irrigation, where for a four-month summer period temperatures are high, and where no organized mosquito abatement practices are in effect. In 1949, none of these factors had been changed. On entering the Valley it was noted that the innumerable permanent and semipermanent bodies of water, a most suitable environment for *C. tarsalis*, were still present.

As soon as collecting was begun, it was obvious that the mosquito population had changed materially since the earlier studies. Certain data may be used to illustrate these changes. New Jersey light traps had been used in 1942 as one technique for sampling the

16. Hammon, W. McD., Reeves, W. C., Brookman, B. and Gjullin, C. M. 1942, Mosquitoes and encephalitis in the Yakima Valley, Washington. V. Summary of cases against *Culex tarsalis* Coquillet as a vector of the St. Louis and Western equine viruses. J. Infect. Dis. **70**: 278–283.

17. Hammon, W. McD., Tigertt, W. D., Sather, G. and Schenker, H. 1949, Isolations of Japanese B encephalitis virus from naturally infected *Culex tritaeniorhynchus* collected in Japan. Am. J. Hyg. **50**: 51–56.

18. Reeves, W. C., Herold, R. C., Rosen, L., Brookman, B. and Hammon, W. McD. Studies on avian malaria in vectors and hosts of encephalitis in Kern County, California. II. Infections in mosquito vectors. To be published.

TABLE 7.—*Number of female mosquitoes collected by New Jersey light traps, Yakima Valley, Washington, first two weeks in August, 1942 and 1949.*

Species	Total collected		Mean number per trial		Median number per trial	
	1942*	1949*	1942*	1949*	1942*	1949*
Culex tarsalis	2404	504	109.0	26.5	67.5	6.0
Culex pipiens	100	33	4.5	1.7	3.0	1.0
Culiseta spp.	413	9	18.8	0.5	10.0	0.0
Anopheles spp.	277	0	12.6	0.0	5.5	0.0
Aedes dorsalis	499	26	22.7	1.4	2.0	0.0
Aedes vexans	164	0	7.5	0.0	2.0	0.0

* Twenty-two trials in 1942 and 19 trials in 1949.

mosquito population. In the 1949 survey, similar traps were run in the same areas. Table 7 illustrates the marked differences in average nightly collections for the first two weeks of August in the two years. In 1942, the mean number of *C. tarsalis* collected per night was 109 and the median 67.5, while in 1949 the mean was only 26.5 and the median 6.0. Other species, such as *C. pipiens*, *Anopheles freeborni* Aitken, *Aedes dorsalis* (Meigen), and *Culiseta incidens* (Thomson), had conspicuously decreased in numbers in the 1949 collections as compared with those of 1942.

Comparisons of records of adults collected in shelters for August, 1942, 1944, and 1949 show the same change (table 8). These figures might be interpreted as an indication that the numbers found in 1949 were still relatively high, since a mean of 65 and a median of 29.5 *C. tarsalis* were found in the average collection. This interpretation is not justified, however, as the data do not reflect the innumerable searches of shelters in which no mosquitoes could be found in 1949 (negative collections were excluded from the study). In 1941, 1942, and 1944, it was unusual to find premises on which mosquitoes could not be found in shelters.

Although irrigation practices and availability of water had not changed, close inspection of flooded pastures, borrow pits, dredger pits, roadside ditches, river sloughs, tule sloughs, waste irrigation water collections, etc., rarely led to discovery of mosquito larvae.

Several persons suggested that the general reduction in mosquitoes (and flies) resulted from widespread application of DDT as an agricultural insecticide. Insofar as could be determined, no effort had been made to gather the facts and to evaluate the situation critically.[19]

The Yakima Valley is a very fertile area with between 400,000 and 500,000 acres of irrigated land. Of this total,

19. Reeves, W. C. 1950, Yakima, Washington, controls mosquitoes and flies at no cost—why can't we? Proc. and Papers 18th Ann. Calif. Mosq. Control Assoc. pp. 13–15.

TABLE 8.—*Number of female mosquitoes collected in domestic shelters, Yakima Valley, Washington, August, 1942, 1944 and 1949.*

Species	Total collected			Mean number per collection			Median number per collection		
	1942*	1944†	1949‡	1942*	1944†	1949‡	1942*	1944†	1949‡
Culex tarsalis	1976	1570	2944	141.1	174.4	65.0	97.5	172.0	29.5
Culex pipiens	158	114	376	11.3	12.7	8.4	2.5	11.0	2.0
Culiseta spp.	241		59	17.2		1.3	3.0		0.0
Anopheles spp.	1382		218	98.7		4.8	87.5		0.0

* Fourteen collections made in period 8-3-42 through 8-20-42.
† Nine collections made in period 8-14-44 through 8-19-44.
‡ Forty-five collections made in period 8-1-49 through 8-15-49.

over 50,000 acres are planted with fruit trees and 60,000 acres are in small field crops. These are sprayed with insecticide each summer to control a variety of plant pests.

In earlier years the standard spray for fruit trees in the area was lead arsenate. Total sales in the area were between four and six million pounds per year from 1940 to 1946. However, after 1945, DDT quickly replaced lead arsenate as the insecticide most frequently used. In addition, DDT is being applied as an insecticide to large acreages of other crops. In the fiscal year 1948–49, 1,224,408 pounds of 50% DDT dust and 368,250 gallons of 5% DDT spray were purchased for local application. In 1945, the first year DDT was used, total purchases were only 700 pounds (table 9).

If the amount of DDT used in the fiscal year 1948–49 had been distributed over the entire irrigated area, more than two pounds of 50% DDT dust and a gallon of 5% spray would have been used on each acre. Of course, the distribution was not equal. As an example, the 33,000 acres of apples and pears received approximately 20 pounds of 50% dust per acre. Needless to say, pastures and other areas between orchards received a marked share of insecticide by drift.

In areas of the Valley where orchards were widespread and where mosquitoes had been particularly abundant in earlier years, either none or very few could be found in 1949 by any means of collecting. In areas of the Valley where pastures and unsprayed crops predominated, relatively large numbers of mosquitoes could still be found.

In addition to DDT, other insecticides of known or potential toxicity to mosquitoes are used widely. In the fiscal year 1948–49, the following purchases were made: benzene hexachloride, 71,032 pounds; parathion, 16,258 pounds; hexaethyl tetraphosphate, 615,700 pounds of dust and 4000 gallons of liquid spray.

TABLE 9.—*Sales of agricultural insecticides, Yakima Valley, Washington, 1940–1948.*

Year	Pounds sold	
	Lead arsenate	50% DDT dust
1940	4,913,237	0
1941	5,000,000	0
1942	5,000,000	0
1943	4,500,000	0
1944	3,500,000	0
1945	3,942,000	700
1946	6,120,663	673,024
1947	92,335	2,612,620
1948	24,640	1,224,808

It was concluded that the marked reduction in the numbers of mosquitoes in the Yakima Valley was due largely to extensive agricultural application of heavy doses of insecticides toxic to mosquitoes. This is strictly an accidental by-product of an unrelated program and was achieved at no additional cost or effort. However, the results are most valuable because they throw light on the potential effect of mosquito control on the epidemiology of the viral encephalitides.

If *C. tarsalis* is the principal vector of the viral encephalitides in the area, a marked wane in the population of this mosquito would decrease the incidence of virus infection in the animal hosts and the vector. Data from the present study indicate that this occurred.

Mites

In the search for arthropod vectors of the viral encephalitides, other than mosquitoes, mites have received considerable attention in recent years.[20–22]

20. Smith, M. G., Blattner, R. J., Heys, F. M. and Miller, A. 1948, Experiments on the role of the chicken mite, *Dermanyssus gallinae*, and the mosquito in the epidemiology of St. Louis encephalitis. J. Exper. Med. **87**: 119–138.

21. Hammon, W. McD. 1948, The arthropod-

While the evidence that mites are of importance as vectors in the Pacific Coast states[10,21,22] is far from conclusive, it seemed advisable to study them.

During 1944, a sample of over 80,000 chicken mites had been collected from chicken houses in the Yakima Valley. These were negative when tested for viruses.[6] During the same period, *C. tarsalis* collected from the same chicken houses were infected with Western equine and St. Louis viruses.[6]

In the present study, mite ectoparasites of chickens and English sparrows were examined. Details of biological data will be presented in another paper.[23] It is interesting that there was no evidence that insecticide spraying of agricultural crops had materially affected the mite population on the species of birds under study.

Thirteen mite collections were made from chicken houses, and from these, 11,816 mites were frozen for testing. All tests for encephalitis viruses were negative. Identification of 1282 mites from these collections indicated that the population was composed of approximately 70% *Dermanyssus gallinae* (De Geer) and 30% *Dermanyssus americanus* Ewing.

Thirty mite-infested English sparrow nests were collected and a sample of 16,077 mites was taken for virus tests. All tests were negative. A sample consisting of 1781 mites was identified; of this number 1777 were *D. americanus* and 4 were *Fonsecaonyssus sylviarum* (C. and F.).

No additional evidence, then, was found to incriminate mites as vectors or reservoirs of the viral encephalitides. If mites should be involved in the natural biological cycle in the absence of mosquitoes, this would have been an exceptionally favorable set of circumstances in which to demonstrate their fundamental role as vectors and reservoirs of the encephalitis viruses. If they were involved, however, it was in a manner which present techniques did not expose.

DISCUSSION

The success of specific control measures against the arthropod-borne viral encephalitides of North America would most logically depend on a successful attack on a critical link in the infection chain. Although vaccination of clinically susceptible hosts such as man and horse is possible, it offers little long-range promise because these hosts apparently are not essential sources of virus for vectors, but rather are accidental entries into a clinically inapparent cycle of virus infection in avian hosts and bloodsucking arthropod vectors. In most instances, morbidity rates in man do not justify mass vaccination for individual protection alone. An attack on the infection in the avian hosts appears equally hopeless because of the large population involved and the inability or infeasibility of efforts directed against wild species.

Epidemiologically, one would expect a reduction in mosquito vector population to be reflected in two ways in the infection cycle: First, as the number of vectors dwindles, the chances are lessened for successful transmission from bird to bird or for infection of the vectors. Second, the lower the population of *C. tarsalis*, and the lower the virus infection rate in this vector, the fewer are the chances for an infected mosquito to feed on a susceptible person or horse. Since these hosts are not the preferred

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borne virus encephalitides. Am. J. Trop. Med. **28:** 515–525.

22. Reeves, W. C. 1951, The encephalitis problem in the United States. Am. J. Pub. Health, **41**(6): 678–686.
23. Doetschman, W. H. Unpublished data.

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